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The epidemiological and evolutionary dynamics of porcine reproductive and respiratory syndrome (PRRS) virus

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV), a member of the family Arteriviridae, encompasses two genetically and antigenically distinct genotypes (type 1 and type 2 PRRSVs). To date, one or both types of PRRSV have been detected in almost all swine-producing countries. In addition, the recent emergence of a highly pathogenic type 2 PRRSV with high mobility and mortality in China has placed neighboring countries at risk. Because understanding the spatial-temporal transmission patterns and methods of pathogen genetic variation is important for effective prevention and vaccine development, the epidemiological and evolutionary dynamics of PRRSV were explored.

The first part of this study focused on the molecular epidemiology of type 1 PRRSV worldwide. Throughout the Bayesian phylogeographical analysis of a large ORF5 dataset collected from 1991 to 2012, details of the diffusion histories of type 1 PRRSV across pig-producing countries were uncovered. Following that phylogeographical footprint, it was found that type 1 PRRSV tended to cluster by geographical locations to form distinctive population structures. In each population, the evolution of the ORF5 gene was best described by a non-homogeneous process.

In the second part of this study, the question of how type 1 PRRSV had evolved upon its introduction to the pig populations on a country level was addressed by focusing on the long-term circulation of the virus in Korea. The maximum likelihood phylogenetic analysis performed on large, worldwide ORF5 sequences strongly suggested no further introduction of genetically novel type 1 PRRSV into Korean pig farms, with the identification of only two clusters in circulation to date. Utilizing a codon-based extension of the Bayesian relaxed clock model, and the novel Bayesian birth-death skyline plot, it was revealed that genetically different clusters of Korean type 1 PRRSV experienced a unique evolutionary and epidemiological dynamics.

Finally, this study investigated and compared the evolutionary dynamics between the highly pathogenic clade and the typical clade of type 2 PRRSV. After applying a codon-based extension of the Bayesian relaxed clock model and the fixed effects maximum likelihood method to all of known structural envelope

protein-coding genes, we were able to demonstrate that the highly pathogenic clade did not display rapid evolutionary dynamics compared with typical type 2 PRRSV. In contrast, several structural genes and codons had been found to evolve in qualitatively different manners and were differentially selected between the typical clade and the highly pathogenic clade of type 2 PRRSV.

In conclusion, this study successfully reconstructs the global transmission histories, the evolutionary and epidemiological dynamics at a country level of type 1 PRRSV. Extended to the genome-scale analysis of evolutionary dynamics, several structural genes and codons have been found to contribute to the differential evolutionary dynamics between the typical clade and the highly pathogenic clade of type 2 PRRSV. The detailed knowledge about the evolutionary and epidemiological dynamics of PRRSV presented in this study yields valuable information in vaccine development, such as which virus strain should be selected for vaccination against type 1 PRRSV in Korea, and which genes should be considered in the application of molecular-based approaches for developing more effective vaccines.

Key words: Porcine reproductive and respiratory syndrome virus, epidemiological dynamics, evolutionary dynamics

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ABBREVIATIONS

AI	Association index
BF	Bayes factors
BIC	Bayesian information criterion
BSSVS	Bayesian stochastic search variable selection
CTMC	Continuous-time Markov chain
E[N]	Absolute non-synonymous substitution rate
E[N|i]	Posterior non-synonymous substitution rate at site i^{th}
E[S]	Absolute synonymous substitution rate
E[S|i]	Posterior synonymous substitution rate at site i^{th}
MC	The maximum monophyletic clade
MCC	Maximum clade credibility
MCMC	Markov chain Monte-Carlo
Nsp	Nonstructural protein
ORF	Open reading frame
PRRS	Porcine reproductive and respiratory syndrome
PRRSV	Porcine reproductive and respiratory syndrome virus
PS	The parsimony score
RT-PCR	Reverse transcription- Polymerase chain reaction
T_{MRCA}	Time to the most recent common ancestor
UTR	Un-translated region

GENERAL INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) encompasses two genetically and antigenically distinct genotypes: type 1 and type 2 PRRSVs (Murtaugh et al., 1995, Nelsen et al., 1999). After it emerged nearly at the same time in the early 1990s in Europe and North America, this virus has spread rapidly; to date, one or both types of PRRSVs have been detected in almost all swine-producing countries.

Only subtype 1 of type 1 PRRSV had been spread worldwide from Europe (Stadejek et al., 2013), and it has been shown to differ from its progenies in terms of antigenic properties (Thanawongnuwech et al., 2004), genetic diversity (Chen et al., 2011, Ropp et al., 2004), and evolutionary trends (Lee et al., 2010). In Korea, since its first report, type 1 PRRSV has been distributed widely among pig farms in the mainland (Lee et al., 2010), co-existed with type 2 PRRSV in the same herds (Kim et al., 2011b), been found in wild boars (Choi et al., 2012), and formed distinct clusters from other subtype 1 PRRSV strains (Kim et al., 2010).

Regarding type 2 PRRSV, the genetic diversity of this genotype has increased rapidly over a short period (Shi et al., 2010b). An example of the rapid evolution is the emergence of the highly pathogenic type 2 PRRSV in China, which was the result of local diversification, leading to increased virulence (Murtaugh et al., 2010, Tian et al., 2007, Zhou et al., 2009a). Paired genomic comparisons of virulent parental/*in vitro* attenuated vaccine viruses for different strains of highly

pathogenic type 2 PRRSVs indicated that the determinants of viral attenuation were multigenic products from both the structural and nonstructural genes (An et al., 2011, Leng et al., 2012c).

With the aim of contributing to the understanding of viral transmission and evolution, this study was designed to reveal the epidemiological and evolutionary dynamics of type 1 and type 2 PRRSV. Chapter I aims to trace the spread of type 1 PRRSV across pig-producing countries. The question of how the virus evolved upon its introduction to the pig population is addressed at the country level for the example of Korean type 1 PRRSV, as described in chapter II. Finally, after genome-scale analysis, chapter III elucidates which gene(s) and codon(s) might be involved in the differences in evolutionary patterns between typical and highly pathogenic type 2 PRRSV.

LITERATURE REVIEW

1. History

Porcine reproductive and respiratory syndrome (PRRS) is characterized by reproductive failure in late gestation sows and respiratory disease in growing pigs (Rossow, 1998). The syndrome was reported for the first time in USA in 1987 (Keffaber, 1989), subsequently described in European countries in early 1990s (Baron et al., 1992, Botner et al., 1994, Paton et al., 1991), and has now emerged in almost all swine-producing countries. The syndrome was occasionally reported as an atypical form, represented by “acute PRRS” in United State in 1996 (Bush et al., 1999), PRRSV-associated reproductive problems in Denmark in 1996 (Madsen et al., 1998, Nielsen et al., 2001), and “high fever” disease in China and Vietnam since 2006 (Feng et al., 2008, Tian et al., 2007).

2. Taxonomy

Porcine reproductive and respiratory syndrome virus (PRRSV) was identified for the first time in 1991 after Koch's postulates were fulfilled (Terpstra et al., 1991, Wensvoort et al., 1991). PRRSV is an enveloped, single stranded, positive sense RNA virus (Benfield et al., 1992, Conzelmann et al., 1993). Together with equine arteritis virus, mouse lactate dehydrogenase elevating virus, and simian hemorrhagic fever, PRRSV is classified within the family Arteriviridae (Snijder and Meulenberg, 1998).

3. Genome organization and biological structure

The PRRSV genome is a polycistronic, positive sense RNA which contains a 5′ un-translated region (UTR) (Snijder and Meulenberg, 1998), and a 3′ UTR which the poly(A) tail is attached (Meulenberg et al., 1993). The viral genome of approximately 15kb in size (Benfield et al., 1992, Meulenberg et al., 1993) consists of ten known open reading frames (ORF). Two large ORF1a and ORF1b genes encode nonstructural proteins (nsp) that are required for virus replication (Allende et al., 1999). Starting from the incoming genome, replicase ORF1a and ORF1b are both expressed into two large precursor polyproteins, pp1a and pp1ab (Fang and Snijder, 2010, Snijder and Meulenberg, 1998). By the autoproteolytic process, pp1a yields 10 end products, including nsp1α, nsp1β, nsp2-nsp6, nsp7α, nsp7β, and nsp8 (Fang and Snijder, 2010, Snijder and Meulenberg, 1998, Ziebuhr et al., 2000). Several nsps were demonstrated to play roles in (i) polyprotein processing (nsp4), (ii) RNA synthesis (nsp9, nsp10) as well as (iii) immune modulation, such as: nsp1, nsp2, nsp7, and nsp11 (Beura et al., 2010, Brown et al., 2009, Chen et al., 2010). Located at the 3’ end of the viral genome are the overlapping genomic regions which code for envelope and nucleocapsid proteins in the order 5’-ORF2a-ORF2b-ORF3-ORF4-ORF5a-ORF5-ORF6-ORF7-3’ (de Lima et al., 2009, Johnson et al., 2011, Snijder and Meulenberg, 1998, van Nieuwstadt et al., 1996, Wu et al., 2001). All of envelope proteins are essential for the production of infectious virus (Sun et al., 2013, Wissink et al., 2005), however, the assembly of viral particles is dependent on both major envelope GP5

and M proteins (Wissink et al., 2005). Several interactions of envelope proteins of PRRSV are known, such as disulfide-linked heterodimers GP5-M proteins (Mardassi et al., 1996), non-covalent heterotrimers GP2-GP3-GP4 proteins (Das et al., 2010, Wissink et al., 2005). Interactions that involved in the entry of virus to susceptible cells are also uncovered between (i) M/GP5 glycoprotein complex and sialoadhesin (Van Breedam et al., 2010), (ii) CD163 with GP2 and GP4 (Das et al., 2010). Recently, an alternative entry receptor was suggested to be present (Frydas et al., 2013).

4. Genetic diversity

Though emerged nearly at the same time in the early 1990's in Europe and North American, and induces similar disease symptoms, PRRSV belongs to two genetically distinct genotypes: type 1 (European type) and type 2 (North American type) PRRSVs (Murtaugh et al., 1995, Nelsen et al., 1999). Type 1 PRRSV was thought to form three major clades, Lelystad-like, Italian-like and Danish (Forsberg et al., 2002). With the recovery of exceptionally diverse isolates from Eastern Europe (Stadejek et al., 2006, Stadejek et al., 2002) and Asian regions of the Russian Federation (Stadejek et al., 2008), a division of type 1 PRRSV into three resolving subtypes was recognized: pan-European subtype 1 (EU/1), and Eastern European subtypes 2 and 3 (EU/2, EU/3). With a recent update, the fourth subtype of type 1 PRRSV was proposed (Stadejek et al., 2013). In another classification proposal, pan-European subtype 1 was divided into 12

different clades, from A to L (Shi et al., 2010a). Type 2 PRRSV was divided into 9 lineages based on an analysis of 8,624 ORF5 sequences collected worldwide (Shi et al., 2010b).

Several factors were suggested contributing to the rapidly expanding genetic diversity of PRRSV over a short period. It was (i) an extrinsic factor of changes in swine husbandry practices in the latter 20^{th} century which provided the virus opportunities for efficient transmission and growth in its host (Murtaugh et al., 2010), and (ii) the ability of the virus to evolve at the high rate on the order of 10^{-3} nucleotide substitutions/site/year (Forsberg, 2005, Shi et al., 2010b, Song et al., 2010). However, an oppose of a tendency for heterogeneity to increase with time (Prieto et al., 2009) was reported for type 1 PRRSV of which very closely related viruses were found over time (Greiser-Wilke et al., 2010, Stadejek et al., 2008). Additionally, processes of natural recombination (van Vugt et al., 2001, Yuan et al., 1999) and "quasispecies" (Chang et al., 2002, Goldberg et al., 2003, Rowland et al., 1999) were known contributing to the genetic variation of PRRSV.

Of a complete genome comparison, type 1 and type 2 PRRSV showed only 63.4% of nucleotide identity (Allende et al., 1999). Within genotype 1, it was found that the genes encode for nonstructural proteins (nsp1, nsp2) and envelope glycoproteins (GP3, GP4, and GP5) exhibited high genetic diversity of nucleotide substitutions and deletions (Darwich et al., 2011, Ropp et al., 2004). Similar conclusions could be reach with the complete genome characterization of a East European subtype 3 (Van Doorsselaere et al., 2012). Interestingly, the relatively

conserved ORF7 gene (Le Gall et al., 1998) were also demonstrated harboring a high level of genetic variation (Jackova et al., 2012, Stadejek et al., 2008). Within genotype 2, the most variable regions in the genome were found to lie within the nsp7-alpha, nsp7-beta of ORF1a, ORF5 and the 3' UTR (Brockmeier et al., 2012).

5. Antigenic diversity

Type 1 and type 2 PRRSV are antigenically distinct genotypes. In early studies, the antigenic heterogeneity between European and American isolates was experimentally demonstrated by the usages of polyclonal antisera (Wensvoort et al., 1992) and monoclonal antibodies against viral structural proteins (Drew et al., 1995, Nelson et al., 1993). Not only structural proteins (Katz et al., 1995, Magar et al., 1997, Meulenberg et al., 1998, Nelson et al., 1993, Pirzadeh et al., 1998) but also non-structural proteins (Brown et al., 2009) were demonstrated that they corresponds to the differences in antigenicity between genotype 1 and genotype 2 of PRRSV. Within each genotype, the antigenic relatedness was also variable (Cheon and Chae, 2000, Frossard et al., 2012, Karniychuk et al., 2010, Yang et al., 1999). Interestingly, the antigenic variability of PRRSV was also known to be affected by *in vivo* passaging (Le Gall et al., 1997).

6. Neutralizing antibodies and their roles

Upon infection, anti-PRRSV antibody responses were detected early (7-9 days post infection) and are non-neutralizing (Lopez and Osorio, 2004). Virus

neutralizing antibody titers > 8 were not detected until 3 to 4 weeks post infection (Loemba et al., 1996). Several structural proteins of PRRSV were known to induce neutralizing antibodies, such as: GP3, GP4, GP5, and M proteins (Cancel-Tirado et al., 2004, Gonin et al., 1999, Weiland et al., 1999, Wissink et al., 2003, Yang et al., 2000). Mostly done with type 2 PRRSV, intensive studies had been carrying out to investigate the role of GP5 in eliciting neutralizing antibodies (Plagemann, 2004, Weiland et al., 1999, Wissink et al., 2003, Yang et al., 2000). Study of (Ostrowski et al., 2002) identified a neutralizing epitope and a nearby non-neutralizing epitope in the ectodomain of GP5. It was hypothesized that during infection with PRRSV, non-neutralizing epitope acted as a decoy, eliciting most of the antibodies directed to GP5, thus delayed the induction of neutralizing antibodies against neutralizing epitope (Ostrowski et al., 2002). Besides, N-glycan shielding of both GP3 and GP5 was uncovered corresponding to a weak and deferred neutralizing antibodies response in pigs (Vu et al., 2011).

Through passive transfer experiments, followed by viral challenge, PRRSV neutralizing antibodies could fully protected sows from reproductive failure, conferred sterilizing immunity in sows and offspring (Osorio et al., 2002). In young weaned pigs, the protection of neutralizing antibodies was dose dependent: a titre of ≥ 1/8 protected piglets against the development of viraemia, a sterilizing immunity was attained at titres of 1/32 (Lopez et al., 2007). Differences in susceptibility to neutralization were reported for both type 1 and type 2 PRRSV. For type 1 PRRSV, genetically unrelated isolates differed in their susceptibility to

neutralization, and no correlation was found between sequences of neutralizing epitopes or the number of N-linked glycosylation sites in GP3, GP4 and GP5 with the neutralization phenotype (Martinez-Lobo et al., 2011). For type 2 PRRSV, the changes of amino acid sequences at three sites of the N-terminal ectodomain of ORF5 of type 2 PRRSV were claimed to affect the susceptibility of field isolates to neutralizing antibodies (Kim et al., 2013).

7. Transmission and persistence infection

PRRSV is able to transmit vertically with the greatest likelihood during the third trimester of pregnancy (Kranker et al., 1998). The horizontal transmission of PRRSV occurs in either of direct or indirect routes (Wills et al., 1997). Under experiment condition, the infectiousness of inoculated pigs was time-dependent that increased from 7 to 14 days post-inoculation (dpi) and then decreased slowly until 42 dpi (Charpin et al., 2012). Also, there was a proportion of inoculated pigs that become persistently infected for several months (Wills et al., 2003). The within herd transmission dynamics suggested that fade-out of virus was most likely to occur within breeding females before virus reached young stock, and persistence was more likely once PRRSV was present in piglets which in turn infected rearing-pigs (Evans et al., 2010).

8. Vaccination and vaccine development

Vaccinated pigs against PRRSV were not protected from infection but could help reducing viraemia, viral shedding (Linhares et al., 2012, Nielsen et al., 1997), as well as clinical signs (Martelli et al., 2009). Experiments of challenging pre-immunized pigs with either homologous strain (Lager et al., 1997) or heterologous strain (Prieto et al., 2008) did not confer complete protection. Recently, it was reported an interesting experimental study that immunization with an attenuated type 1 PRRSV vaccine provided partial protection (reducing both of weight loss and development of clinical signs) against challenge with a highly pathogenic type 2 PRRSV strain (Roca et al., 2012). Carrying out the efficacy assay, it was suggested that modified live virus vaccine was the only type of vaccine capable of establishing protective immunity, while killed vaccine evoked no measurable protective immunity (Zuckermann et al., 2007). And that the protection induced by the modified live vaccine did not appear to be based on humoral but rather on cell-mediated immunity (Zuckermann et al., 2007). Several studies showed that the efficacy of vaccine did not tightly relate to the genetic similarity of ORF5 gene between vaccine strain and challenge isolate (Diaz et al., 2006, Martelli et al., 2009). It was suggested that the ability of each strain to induce a strong cell-mediated immune response was more important than the genetic similarity inducing protection (Mateu and Diaz, 2008). Toward develop a vaccine against the highly pathogenic type 2 PRRSV, several studies attempted to produce attenuated strain by high passage of the parental strain and showed to

confer protection against a lethal wild-type challenge (Leng et al., 2012b, Tian et al., 2009). Recently, among efforts on PRRSV vaccine development, DNA shuffling approach was applied to produce chimeric virus by either ORF3, ORF4 or ORF6 gene shuffling from different strains (Zhou et al., 2013, Zhou et al., 2012). The replication ability of the chimeras both *in vitro* and *in vivo* was not impaired by gene shuffling. Importantly, higher level of cross-neutralizing antibodies could be induced (Zhou et al., 2013, Zhou et al., 2012).

9. Genetic-based diagnosis of PRRSV

Samples used to detect nucleic acid of PRRSV included semen, serum, peripheral blood mononuclear cells, and tissues (Christopher-Hennings et al., 2001). For detecting persistently PRRSV infected pigs, oropharyngeal scrapings was more proper than tonsil homogenates (Horter et al., 2002). Recently, oral fluid was suggested to be suitable for surveillance of PRRSV prevalence (Olsen et al., 2013) and PRRS serology (Kittawornrat et al., 2012). To date, numbers of RT-PCR based methods had been developed to simultaneously detect or differentiate both type 1 and type 2 of PRRSV (Kleiboeker et al., 2005, Kono et al., 1996, Li et al., 2009, Martinez et al., 2008). Tests were also available for rapid differentiation between type 1, typical and highly pathogenic type 2 PRRSV (Chai et al., 2013, Wernike et al., 2012). However, one should be aware of genetic variants which might occur in the primer or probe-binding sites that potentially yield false-negative results (Toplak et al., 2012, Truyen et al., 2006). It was shown for ORF7

gene that mutations were frequently affecting 3' end and central primer regions (Stadejek et al., 2008). Nowadays, the diagnosis and genotyping of PRRSV could be based on full genome sequences which relied on next generation sequencing technique (Kvisgaard et al., 2013) or sequence-independent PCR technique (Van Doorsselaere et al., 2011).

10. Bayesian coalescent-based Markov chain Monte Carlo method

Measurably evolving populations (MEPs) are characterized by either a high mutation rate, or a wide range of sequence sampling times (Drummond et al., 2003). For nearly all RNA viruses, overall rates are in the range of 10^{-2} to 10^{-5} nucleotide substitutions/site/year (Duffy et al., 2008), thus RNA viruses are recognized as a primary source of MEPs (Drummond et al., 2003). The analysis of MEPs requires integrations of temporally spaced sequences, phylogenetic and coalescent models (Drummond et al., 2003). Bayesian statistical framework, such as BEAST (Drummond and Rambaut, 2007), is suitable to study microevolutionary processes of MEPs. The models specified in a BEAST analysis contains several components: (i) a model of nucleotide substitution, such as a codon-based SRD06 which was shown to provide a better fit for protein coding sequences (Shapiro et al., 2006); (ii) a strict or relaxed molecular clock to model the distribution of rates among branches; (iii) and a demographic model to describe the change in effective population size through time.

Several key applications of Bayesian inference to explore microevolution of MEPs are reconstructions of past population dynamics, spatiotemporal diffusion dynamics, and epidemic spread. The Bayesian skyline plot which allows for both constant and complex changes in population size over time, was demonstrated as a powerful method to discover demographic signatures (Drummond et al., 2005). By a Bayesian phylogeographic analysis (Lemey et al., 2009), historical dispersal patterns of a virus could be inferred, visualized and tested for significance of diffusion rates. As an alternative to the coalescent-based skyline plot, a recently developed birth–death skyline method (Stadler et al., 2013) models epidemiological transmission as a birth-death process. This method relaxes the assumptions of constant transmission rate, constant noninfectious rate, and constant sampling probability by allowing these parameters to change in a piecewise, constant fashion. Thus, it enables the estimation of temporal changes of effective reproductive number.

11. Tracing the evolutionary histories of PRRSV by Bayesian analysis

In addressing the genetic history derived from the worldwide, complete ORF5 to ORF7 sequences of type 1 and type 2 PRRSV, a Bayesian coalescent approach inferred that PRRSV evolving at the rates of 1.55×10^{-3} substitutions/site/year, and the time of the most recent common ancestor for type 1 and type 2 viruses was 58.7 and 62.6 years ago, respectively (Yoon et al., 2012). Of the type 1 PRRSV circulating in British, a relaxed molecular clock using the ORF7

sequences dated the most recent common ancestor of all British viruses to 1991, and inferred the rate of substitution as 3.8 x 10^{-3} per site per year (Frossard et al., 2012). With the same method, type 1 and type 2 PRRSV circulating in a small scale population were demonstrated undergoing faster evolution than overall PRRSV evolution rate in the world (Kim et al., 2011b). Especially, through the applications of Bayesian phylogenetic and phylogeographic inferences to a large ORF5 dataset, a deep into geographical spread of type 2 PRRSV in North America was made available, in which the patterns of virus flow was uncovered to strong resemble to the hog movements (Shi et al., 2010b, Shi et al., 2013).

Chapter I. A Bayesian phylogeographic analysis of type 1 porcine reproductive and respiratory syndrome virus (PRRSV)

Abstract

Understanding viral transmission is an important factor for the effective prevention one of the most devastating swine diseases, porcine reproductive and respiratory syndrome. Focusing on molecular epidemiology of type 1 PRRSV, this study analyzed a large ORF5 dataset collected worldwide from 1991 to 2012 using a coalescent-based Bayesian Markov chain Monte Carlo approach. The results suggested that the virus diversified into unique subpopulations in Russia & Belarus and Italy approximately 100 years ago. Previously unreported consecutive diffusions of the virus were identified, which showed that some countries, such as Spain and Germany, acted as distribution sources to some extent. This study also provided statistical evidence for the existence of an ORF5-based phylogeographic structure, in which type 1 PRRSV tended to cluster by geographical locations more tightly than expected by chance. In contrast to this tight geographic structure, based on mapping of non-synonymous per synonymous substitutions, the evolution of the ORF5 gene was best described by a non-homogeneous process that could be implicated as a mechanism for viral immune evasion.

1.1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is recognized as one of the most important viral pathogens in swine. PRRSV induces reproductive failure in late gestation sows and respiratory disease in growing pigs (Rossow, 1998). The syndrome was occasionally reported as an atypical form, represented by “acute PRRS” in United State in 1996 (Bush et al., 1999), and “high fever” disease in China and Vietnam since 2006 (Feng et al., 2008, Tian et al., 2007). The disease causes devastating economic damage to the pig industry; for example, it is responsible for approximately $560 million in losses per year in the United States (Neumann et al., 2005). The virus encompasses two genetically and antigenically distinct genotypes that produce similar disease symptoms: type 1 (European type) and type 2 (North American type) PRRSVs (Murtaugh et al., 1995, Nelsen et al., 1999). Type 1 PRRSV was thought to form three major clades, Lelystad-like, Italian-like and Danish (Forsberg et al., 2002). With the recovery of exceptionally diverse isolates from Eastern Europe (Stadejek et al., 2006, Stadejek et al., 2002) and Asian regions of the Russian Federation (Stadejek et al., 2008), a division of type 1 PRRSV into three resolving subtypes was proposed: pan-European subtype 1 (EU/1), and Eastern European subtypes 2 and 3 (EU/2, EU/3). In another classification proposal, pan-European subtype 1 was divided into 12 different clades, from A to L (Shi et al., 2010a). Type 2 PRRSV was divided into 9 lineages based on an analysis of 8,624 ORF5 sequences collected worldwide (Shi et al., 2010b). Further investigation of their genetic

variation showed that PRRSVs generate genotype-related quasispecies-specific sequences during natural infection (Chang et al., 2002, Goldberg et al., 2003, Rowland et al., 1999).

From an epidemiological point of view, prior to the first documented outbreak, serological evidence of PRRSV was demonstrated in pig populations in North America (Carman et al., 1995, Yoon et al., 1992), Europe (Ohlinger et al., 2000), and Asia (Shin et al., 1993). To date, one or both types of PRRSVs have been detected in almost all swine-producing countries. Intriguingly, work performed by Stadejek et al. (Stadejek et al., 2006, Stadejek et al., 2008, Stadejek et al., 2002), focused on the distribution of type 1 PRRSV in Europe, uncovered a contrast between a single circulating strain of European subtype 1 in Western Europe and the co-circulation of highly diverse East European subtypes 2, 3 in Eastern European and Russian Federation countries. Outside of Europe, in chronological order, type 1 PRRSV was detected by RT-PCR in Canada (1999) (Dewey et al., 2000), USA (1999) (Ropp et al., 2004), Thailand (2001) (Thanawongnuwech et al., 2004), Korea (2006) (Kim et al., 2006), China (2006) (Chen et al., 2011). Those circulating type 1 PRRSVs were suggested to differ from their progenies in terms of antigenic properties (Thanawongnuwech et al., 2004), genetic diversity (Chen et al., 2011, Ropp et al., 2004), and evolutionary trend (Lee et al., 2010). Based on phylogenetic analyses, genetic relatedness of type 1 PRRSVs between countries were also proposed (Lee et al., 2010, Ropp et al., 2004, Thanawongnuwech et al., 2004). Nevertheless, to our knowledge, there has been

no statistics-based analysis of the transmission patterns of type 1 PRRSV since the first endemic was reported. Using a large database of ORF5 sequences collected worldwide, this study aimed to trace the spread of type 1 PRRSV across pig-producing countries. To this end, the study first investigated the spatial-temporal dynamics of type 1 PRRSV worldwide. Following that phylogeographical footprint, a hypothesis for viral population structure was tested. Finally, an attempt was made to characterize differences in the context of spatial and temporal variations of non-synonymous/synonymous substitution patterns in the ORF5 gene, which encodes an important structural surface protein with roles in particle production (Wei et al., 2012, Wissink et al., 2004), viral neutralization (Ostrowski et al., 2002, Wissink et al., 2003), and other processes.

1.2. Materials and methods

1.2.1. Sample collection

The ORF5 gene, encoding the major envelope glycoprotein GP5, was selected for analysis due to its high genetic variability. The final dataset contained 385 sequences originating from Europe (Italy, Germany, Czech, Netherlands, Spain, Denmark, France, Belgium, UK, Hungary, Portugal, Belarus, and Russia), North America (USA) and Asia (China, Thailand, and Korea), and covering a sampling period from 1991 to 2012. Of the 385 ORF5 sequences analyzed, 11 were generated in this study, and the others were retrieved from GenBank. The year of isolation and sampling location of each sequence were extracted from published works or information available in GenBank. Sequences that lacked information for either isolation date or sampling location were excluded. The details of the dataset are summarized in supplementary Table S1.1.

1.2.2. Bayesian phylogenetic inference

Coalescent-based Bayesian Markov chain Monte Carlo (MCMC) inference was applied to reconstruct the evolutionary tree of type 1 PRRSV, under the assumptions of (i) a codon based SRD06 nucleotide substitution model (Shapiro et al., 2006), (ii) a constant population size for the coalescent prior, and (iii) three available molecular clock models. In each analysis, 8 independent runs (50 million chains, sampling every 10,000 generations) were performed using BEAST

package v1.6.2 (Drummond and Rambaut, 2007), which is available at the Bioportal server (Kumar et al., 2009). The output log files from multiple runs were combined (with proper burn-in steps) using LogCombiner v1.6.2. The combined log files were subsequently analyzed in Tracer v1.5 to assess the convergence (effective sample size > 100) and to calculate Bayes factors (BF) for molecular clock model comparisons. The interpretation of BF follows previous guidelines (Kass and Raftery, 1995): 2>2 $\log_e$(BF)>0, 6>2 $\log_e$(BF)>2, 10>2 $\log_e$(BF)>6, and 2 $\log_e$(BF)>10 indicate no, positive, strong, and very strong evidence against the null model.

1.2.3. Bayesian phylogeographic analysis

A Bayesian framework (Lemey et al., 2009) was applied to reconstruct the spatial-temporal diffusion history of type 1 PRRSVs. In brief, the spatial diffusion of the time-scaled genealogy is modeled as a standard continuous-time Markov chain (CTMC) process over discrete sampling locations. A Bayesian stochastic search variable selection (BSSVS) approach, which allows the exchange rates in the CTMC to be zero with some prior probability, was used to find a parsimonious set of rates explaining the diffusions in the phylogeny. Finally, SPREAD v1.0.4, a cross-platform application (Bielejec et al., 2011) was used to analyze (Bayes factor test) and visualize the Bayesian phylogeographic reconstructions incorporating spatial-temporal diffusion.

1.2.4. Testing for phylogeographic structure

The association between the phylogeny and geography of type 1 PRRSVs, based on ORF5 gene, was investigated using a Bayesian MCMC approach, implemented in Befi-BaTS v0.1.1 (Parker et al., 2008). Accounting for phylogenetic error, the program analyzed the posterior set of trees generated by BEAST package (Drummond and Rambaut, 2007). The presence of phylogeny-trait (sampling location) correlations was assessed using three statistics: parsimony score (PS), association index (AI), and maximum monophyletic clade (MC) size. The null hypothesis of random phylogeny-trait associations was rejected at a significance level of 0.05 (p-value>0.05). The population structure of the virus was further tested by estimating the level of gene flow among type 1 PRRSV populations (categorized by sampling location), indicated by the F_{ST} value (Hudson et al., 1992). F_{ST} was calculated with DnaSPv5 (Rozas et al., 2003).

1.2.5. Characterizing the non-synonymous/synonymous substitution patterns of ORF5 gene

The ORF5 dataset (n=385) was analyzed under a non-homogeneous model of sequence evolution implemented in the TestNH package (Dutheil et al., 2012). In this analysis, branches of the type 1 PRRSV phylogenetic tree were clustered according to their similarity in ratios of nonsynonymous to synonymous codon substitutions (dN/dS) along the tree. The overall procedure could be summarized in the following steps: (i) fitting a homogeneous model with single dN/dS for all

branches, assuming a Yang and Nielsen (YN98) codon substitution model; (ii) clustering branches freely through dN/dS substitution mapping (very short branches were automatically clustered with their parents); (iii) defining and testing partitions with nonhomogeneous models, using Bayesian information criterion (BIC) as the model selection criterion.

1.3. Results

1.3.1. Evolutionary parameters of type 1 PRRSV based on the ORF5 gene

Supported by the Bayes factor test, a strict molecular clock model was rejected in favor of the relaxed molecular clock models (2 $\log_e$(BF) = 301.21). Of the two relaxed molecular clocks, the model of uncorrelated exponential distribution fit the data significantly better than the model of uncorrelated lognormal distribution (2 $\log_e$(BF) = 19.25). Under that assumption, the geometric mean of the time to the most recent common ancestor (T_{MRCA}) of type 1 PRRSV was approximately 107 years ago (95% highest posterior density (HPD) intervals: 40.34 - 231.37). The mean nucleotide evolutionary rate of ORF5 was estimated as approximately 5.815×10^{-3}/site/year (95% HPD intervals: 4.560×10^{-3} - 7.580×10^{-3}).

1.3.2. Bayesian phylogeography of type 1 PRRSVs circulating worldwide

The result of the Bayesian phylogeographic analysis is shown in Figure 1.1. At the root of the type 1 PRRSV phylogeny, there was a separation into two distinct branches, one that split into the Russian & Belarusian strains (EU/2, EU/3, and Russian EU/1) and the other that was comprised of the Italian strains (subtype EU/1). The maximum clade credibility (MCC) phylogeny showed that the two viral populations (circulating in Russia & Belarus and Italy) expanded in different trajectories after this initial separation. The diffusion patterns of worldwide EU/1 PRRSVs along the trunk of evolutionary tree prior to the first detected outbreak in

1990 in Europe (Paton et al., 1991) could be roughly divided into fourth consecutive stages (labeled from (i) to (iv), (Figure 1.1)). During that period, phylogeography captured switches in root location states from Italy to Spain, Spain to Germany, and Germany to Spain. The estimated root state probability distribution indicated that Italy was the probable original location from which all currently circulating subtype EU/1 PRRSVs worldwide spread. All significantly non-zero migration rates supported by Bayes factor (BF≥3) analysis are summarized in Figure 1.2. Accordingly, several European countries, such as Spain and Germany, were inferred act, to some extent, as sources in the viral migration network. With respect to spatial diffusion events outside of Europe, the results demonstrated that type 1 PRRSVs in Asian countries (Thailand, Korea, and China) and North America (USA) originated from different sources. Interestingly, no cross transmission between Asian countries was observed.

1.3.3. Quantifying the phylogeographic structure of type 1 PRRSV

The phylogeny-trait (sampling location) associations of type 1 PRRSVs were quantified using Befi-BaTS (Parker et al., 2008). As shown in Table 1.1, trait association tests (AI and PS) of the phylogeographic structure rejected the null hypothesis of no association between sampling location and phylogeny. Supported by the MC statistic, type 1 PRRSVs tended to cluster by geographical locations more than expected by chance, except for samples from Spain, Netherlands, and

Portugal. Similarly, the estimated F_{ST} value = 0.478 (greater than 0) indicated genetic isolation among tested populations.

1.3.4. Non-synonymous/synonymous substitution patterns of ORF5 gene

As shown in Figure 1.3, the free clustering approach suggested 7 partitions with similar dN/dS substitutions along the ORF5-based type 1 PRRSV phylogeny (model chosen according to the lowest BIC score = 35765.4). At the deep internal nodes, switches in dN/dS were encountered. It was shown that the dN/dS patterns of the most distant type 1 PRRSV subtypes (EU/2, EU/3 and Russian EU/1) did not differ from those of the worldwide subtype EU/1. That observation also held for within-subtype comparisons. Overall, in contrast to the tight geographic structure (Table 1.1), the trajectories of dN/dS seemed to vary freely in a limited range, regardless of subtype, sampling date and location.

1.4. Discussions

In this study, different aspects of a large ORF5 dataset of type 1 PRRSV collected worldwide were analyzed. Drawn from the data for a best-fit uncorrelated relaxed molecular clock model, the virus was suggested to possess a high evolution rate of 5.815×10^{-3}/site/year. That estimation is within the range of 10^{-2} to 10^{-5} nucleotide substitutions/site/year for nearly all RNA viruses (Duffy et al., 2008) and generally agreed with previous inferences of PRRSV substitution rates (Forsberg, 2005, Song et al., 2010). Unlike previous studies (Forsberg, 2005, Yoon et al., 2012) in which the most diverged type 1 PRRSVs collected from Russia and Belarus were not included for the calculation of T_{MRCA}, using a non-contemporaneous dated-tip, this study was not only able to infer that type 1 PRRSV emerged approximately 100 years ago but also supported the early diversification of type 1 PRRSV into two unique branches/populations (Russian & Belarusian and Italian strains, Figure 1.1). The results were in line with the finding that Russian type 1 PRRSVs were loosely related to the pan-European subtype 1 found worldwide (Stadejek et al., 2008) and that PRRSV had most likely been endemic in Russia and Italy for a long time (Andreyev et al., 2000). Moreover, the Bayesian phylogeographic analysis at global scale (among 17 countries) provided in-depth information regarding the diffusion history of type 1 PRRSV. The virus indeed evolved and was serially transmitted among European countries before the first recorded type 1 PRRSV epidemic in this continent in the early 1990s. Despite unequal numbers of ORF5 sequences originating from

different countries, the Bayesian phylogeographic inference was not biased toward an important role of the countries with more included sequences, such as Korea (n=82), Hungary (n=45), and Thailand (n=44). Importantly, the analysis suggested that some countries (Spain, Germany, for example) acted as key sources in the viral migration network. Prior to this study, it had been speculated that type 1 PRRSVs might have been introduced to Asian pig populations (Chen et al., 2011, Lee et al., 2010, Thanawongnuwech et al., 2004). However, applying the BSSVS procedure (Lemey et al., 2009) allowed us to trace the origin and spread of the virus in detail, and we uncovered complex histories of type 1 PRRSV in each pig population. The dataset used in this study has a limitation due to a lack of sequences from some countries (not in the public database, or missing either collection data or sampling location information) that leaves the picture of the transmission patterns of type 1 PRRSV worldwide incomplete. Thus, further analyses should be carried out as new sequences are made available.

Regarding the geographic distributions of type 1 PRRSV all over the world, this study provided statistical evidence for the existence of phylogeographic structure. Accordingly, it was found that type 1 PRRSV tended to cluster by geographical locations more tightly than expected by chance. This result seemed to contradict the suggestion of poor geographic structuring in the type 1 PRRSV phylogeny (Shi et al., 2010a). That observation was based on defining type 1 PRRSVs into 12 clades and finding that each clade was associated with multiple countries, except for the Italian clades. In contrast, this study viewed the geographic

structure of type 1 PRRSV phylogeny in association with country of origin regardless of genetic classification in clades A to L. Rather, a significant population subdivision of the virus upon introduction to pig populations could be inferred from our analysis. For example, the lack of a phylogeographic structure of type 1 PRRSV in Spain correlated strikingly well with the role of Spain as a viral intermediate distribution source, which was predicted by the Bayesian phylogeographic analysis (Figure 1.1, Figure 1.2).

In this study, which included all type 1 PRRSV subtypes (EU/1, EU/2, and EU/3) consecutively collected from 1991-2012 over 17 countries, it was desirable to characterize the ORF5 dN/dS substitution patterns in the context of spatial and temporal variations. We chose to use the fast and robust (Romiguier et al., 2012) substitution mapping (mapping the history of nucleotide or amino-acid changes onto the phylogenetic tree) method. By applying a novel clustering algorithm (Dutheil et al., 2012), it was possible to investigate the dN/dS substitution patterns of ORF5 gene in large scale without relying on subjective assumptions. The variations in dN/dS observed within and among type 1 PRRSV subtypes might be intrinsically derived from the ability of the virus to simultaneously generate multiple variants on farms and within individual animals during natural infection (Chang et al., 2002, Goldberg et al., 2003). It also reflected the continuous changes in the ORF5 gene, which was demonstrated to be the least conserved gene in the PRRSV genome, containing several variable and conserved regions (Mateu et al., 2006, Music and Gagnon, 2010, Pesch et al., 2005). In nature,

dN/dS can vary among species, genes, or among codons of the same gene. Nevertheless, the significance of our result was in the finding that there were several distinct dN/dS substitution patterns along the ORF5-based type 1 PRRSV phylogeny spanning 21 consecutive sampling years over 17 countries. From this point of view, our results agreed with the suggestion of ORF5 genetic plasticity for type 2 PRRSVs and that a certain variability threshold seems to exist and limits the range of heterogeneity observed in the field over time (Delisle et al., 2012). On the other hand, because multiple clusters of dN/dS substitutions may exist in a given pig population, the variability in ORF5 might be large enough to combat against herd immunity once new variants were introduced.

In conclusion, this study first investigated the spatial-temporal dynamics of type 1 PRRSV worldwide and inferred that the virus existed approximately 80 years prior to the first recorded epidemic and supported the early diversification of type 1 PRRSV into different subtypes. Captured by phylogeographic analysis, details of the diffusion histories of type 1 PRRSV across pig producing countries were uncovered. Following that phylogeographical footprint, a hypothesis for viral population structure was tested which showed that type 1 PRRSVs tended to cluster by geographical location more strongly than expected by chance, with few exceptions. Finally, it was shown that patterns of dN/dS within and among type 1 subtypes did not differ from each other, and the trajectories of dN/dS seemed to vary freely in a limited range, regardless of subtype, sampling date and location.

Table 1.1. Statistical analysis of geographic structuring of type 1 PRRSV based on ORF5 gene

Statistic*	***Observed value***			***Null value***			***p value****
	Mean	*Lower 95% CI*	*Upper 95% CI*	*Mean*	*Lower 95% CI*	*Upper 95% CI*	
AI	0.971	0.499	1.497	38.495	36.769	40.079	**0.000**
PS	47.250	44.000	50.000	331.729	323.750	338.938	**0.000**
MC(Belarus)	8.000	8.000	8.000	1.055	1.000	1.292	**0.010**
MC(Belgium)	2.000	2.000	2.000	1.000	1.000	1.000	**0.010**
MC(China)	4.000	4.000	4.000	1.003	1.000	1.000	**0.010**
MC(Czech)	3.000	3.000	3.000	1.044	1.000	1.083	**0.010**
MC(Denmark)	8.188	7.000	9.000	1.163	1.000	2.000	**0.010**
MC(France)	2.688	2.000	3.000	1.018	1.000	1.000	**0.010**
MC(Germany)	20.750	15.000	23.000	2.096	1.479	3.000	**0.010**
MC(Hungary)	24.000	24.000	24.000	1.938	1.104	2.604	**0.010**
MC(Italy)	34.000	34.000	34.000	2.379	2.000	3.125	**0.010**
MC (Korea)	57.000	57.000	57.000	2.563	2.000	4.000	**0.010**
MC(Netherlands)	1.000	1.000	1.000	1.000	1.000	1.000	1.000
MC(Portugal)	1.000	1.000	1.000	1.000	1.000	1.000	1.000
MC(Russia)	21.000	21.000	21.000	1.294	1.000	2.000	**0.010**

Statistic*	***Observed value***			***Null value***			***p value*****
	Mean	*Lower 95% CI*	*Upper 95% CI*	*Mean*	*Lower 95% CI*	*Upper 95% CI*	
MC(Spain)	1.000	1.000	1.000	1.036	1.000	1.167	1.000
MC(Thailand)	21.000	21.000	21.000	1.876	1.250	2.354	**0.010**
MC(UK)	2.500	2.000	3.000	1.016	1.000	1.000	**0.010**
MC(USA)	17.354	17.000	18.000	1.256	1.000	2.000	**0.010**

** Statistical parameters: the association index (AI), the parsimony score (PS), the maximum monophyletic clade (MC) size*

*** Values in bold indicate statistical significance.*

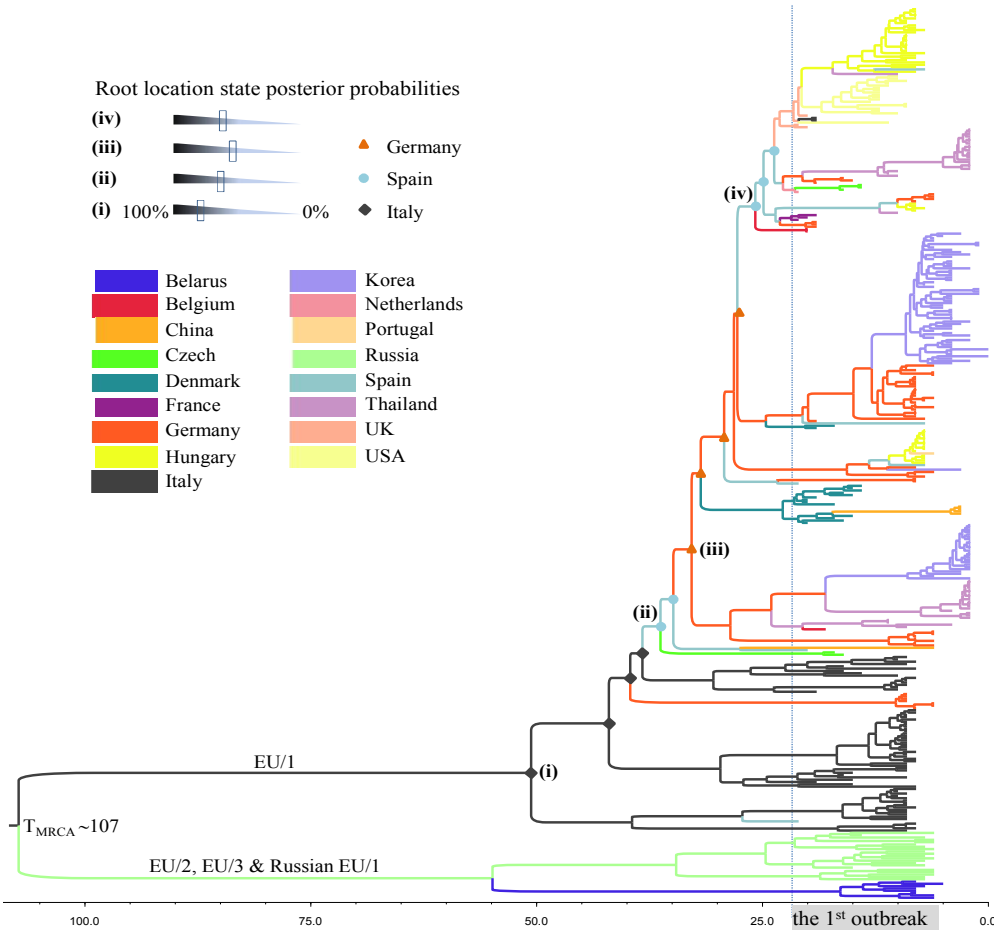


Figure 1.1. Bayesian time-scaled phylogeny of type 1 PRRSV with inferred geographical location states. The branches of maximum clade credibility tree were colored according to the most probable location state of their descendent nodes. The color codes are defined in the insert legend. Prior to the first detected outbreak, phylogeography captured switches in root location states (labeled from (i) to (iv)) from Italy to Spain, Spain to Germany, and Germany to Spain. The time-scale (year) of evolutionary changes represented in the tree is indicated by the scale bar.

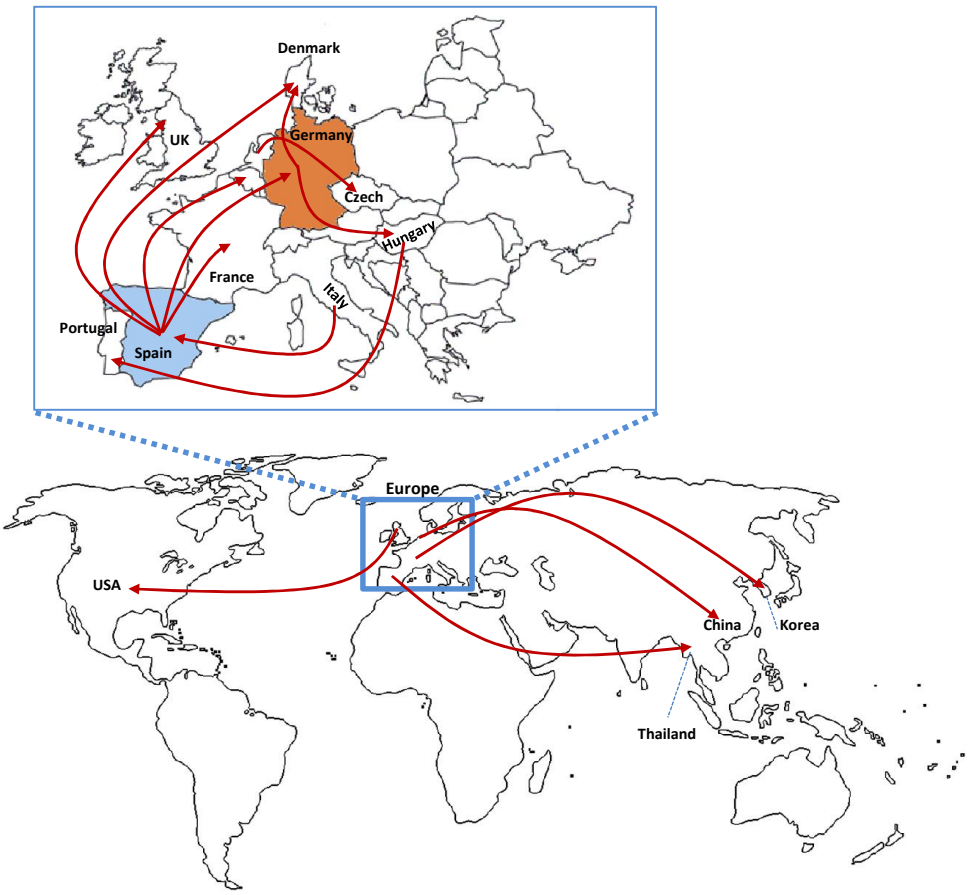


Figure 1.2. Spatial dynamics of type 1 PRRSV, supported by Bayes factor ≥3. The upper panel showed transmission events among European countries. It was implied that Spain acted as central node in the viral migration network. The lower panel indicated spatial diffusion events from Europe to Asian countries (Thailand, Korea, and China) and North America (USA).

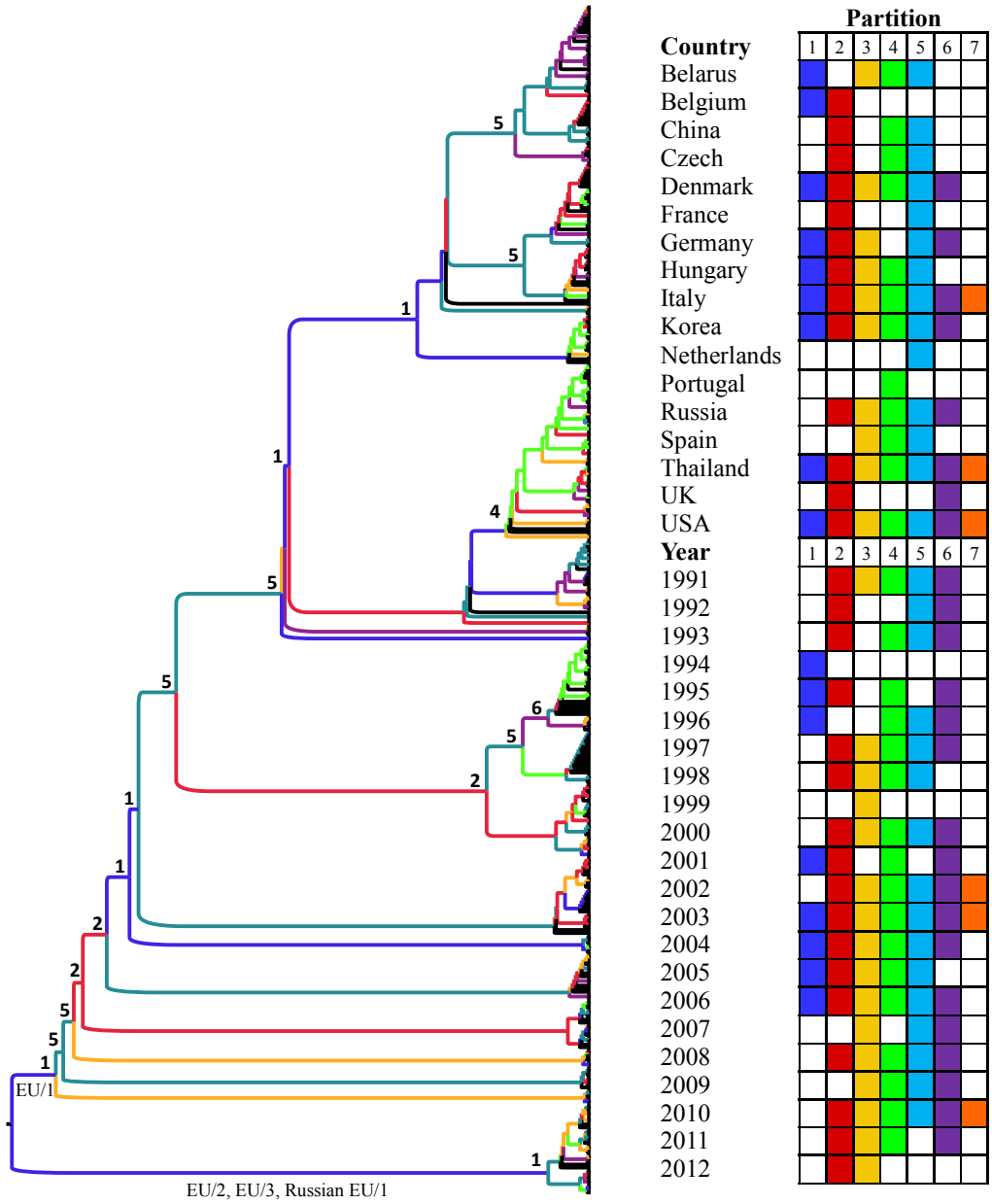

Partition
Country
1 2 3 4 5 6 7
Belarus
Belgium
China
Czech
Denmark
France
Germany
Hungary
Italy
Korea
Netherlands
Portugal
Russia
Spain
Thailand
UK
USA
Year
1 2 3 4 5 6 7
1991
1992
1993
1994
1995
1996
1997
1998
1999
2000
2001
2002
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
EU/1
EU/2, EU/3, Russian EU/1

Figure 1.3. Non-synonymous/synonymous (dN/dS) substitutions patterns of the ORF5 gene of type 1 PRRSV. The free clustering approach suggested 7 partitions with similar dN/dS substitutions along the phylogeny (left panel). Branches with similar dN/dS substitutions were labeled with the same color. The numbers at the nodes of the phylogeny denote the number of partition to which they belong (for clarity, labels for some terminal nodes were omitted). Note that dN/dS varied freely in a limited range (partitions 1-7) and was not correlated with sampling date or location (right panel).

Chapter II. ORF5-based evolutionary and epidemiological dynamics of the type 1 porcine reproductive and respiratory syndrome virus circulating in Korea

Abstract

This study applied a number of advanced genetic analysis tools to investigate the evolutionary trajectories and epidemiological dynamics of Korean type 1 PRRSV based on variations in the ORF5 gene over a long-term period from 2005 – 2013. Maximum likelihood phylogenetic analysis performed on large, worldwide ORF5 sequences (n = 1127) strongly suggested no further introduction of genetically novel type 1 PRRSV into Korean pig farms, with the identification of only two clusters (I and II) in circulation to date. Using a codon-based extension of the Bayesian relaxed clock model, this study was able to distinguish between synonymous and non-synonymous substitutions and demonstrated that, while the absolute rates of synonymous substitution (E[S]) were similar between clusters I and II, the absolute rate of non-synonymous substitution (E[N]) was significantly different between the clusters. Cluster I was found to have an elevated E[N]/E[S] ratio relative to cluster II on the internal branches, compared to the external branches. Additionally, many fewer sites were predicted under diversifying selection in cluster II than in cluster I. Utilizing the Bayesian skyride method and the Bayesian birth-death skyline plot method, this study provided insights into the epidemiological dynamics of type 1 PRRSV in Korea by revealing that each cluster experienced a unique epidemic growth and by uncovering correlations between the effective population size and effective reproductive number.

2.1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV), a member of the family *Arteriviridae*, has a positive-sense, non-segmented, single-stranded RNA genome approximately 15 kb in size (Benfield et al., 1992, Meulenberg et al., 1993). The virus encompasses two genetically and antigenically distinct genotypes (type 1 and type 2 PRRSVs, respectively) (Murtaugh et al., 1995, Nelsen et al., 1999) that induce similar disease symptoms: reproductive failure in late gestation sows and respiratory disease in growing pigs. The disease causes devastating economic damage due to productivity losses in breeding and growing-pig herds (Holtkamp et al., 2013). Regarding its genome organization, PRRSV is known to consist of ten open reading frames (ORFs). Of these, ORF5 (600 – 603 nucleotides) is an essential gene because its encoded product, the major envelope glycoprotein (GP5), is involved in many important processes, such as the production of infectious particles (Wissink et al., 2005), enhanced infection and replication in the presence of specific antibody (Cancel-Tirado et al., 2004), the entry of virus to susceptible cells (Van Breedam et al., 2010), etc. Because of marked genetic variation, this gene had also been used widely in studies on microevolution (Nilubol et al., 2013, Shi et al., 2010b).

Focused on type 1 PRRSV, this genotype was divided into three resolving subtypes: pan-European subtype 1 and Eastern European subtypes 2 and 3 (Stadejek et al., 2008). Type 1 PRRSV (i) diverged prior to the first detected outbreak in 1990 in Europe and (ii) displayed a sharp geographical demarcation of

the single circulating subtype 1 in Western Europe and the co-circulation of the highly diverse subtypes 2 and 3 in Eastern European and Russian Federation countries (Stadejek et al., 2006, Stadejek et al., 2008, Stadejek et al., 2002). Only subtype 1 spread to other continents (migrating from Europe) (Stadejek et al., 2013): it was detected by RT-PCR and reported in Canada (1999) (Dewey et al., 2000), USA (1999) (Ropp et al., 2004), Thailand (2001) (Thanawongnuwech et al., 2004), Korea (2006) (Kim et al., 2006), and China (2006) (Chen et al., 2011). Circulating type 1 PRRSVs have been shown to differ from their progenies in terms of antigenic properties (Thanawongnuwech et al., 2004), genetic diversity (Chen et al., 2011), and evolutionary trends (Lee et al., 2010).

Since its first report in Korea, type 1 PRRSV was distributed widely among pig farms in the mainland (Lee et al., 2010), co-existed with type 2 PRRSV in the same herds (Kim et al., 2011b), was found in wild boars (Choi et al., 2012), and formed distinct clusters from other European genotype PRRSV strains (Kim et al., 2010). Nevertheless, little is known regarding the evolutionary trajectories or the epidemiological dynamics of the virus. Based on the ORF5 gene, this study first revisited and updated the state of type 1 PRRSV in Korea. Then the evolutionary dynamics was uncovered by quantifying (i) the nucleotide substitution rates, (ii) the absolute rate of synonymous and non-synonymous substitutions, (iii) the site-to-site rate patterns, and (iv) the diversifying selective pressures. Finally, we attempted to reconstruct the temporal changes in effective population size and epidemic spread of the virus over a long-term period from 2005 to 2013.

2.2. Materials and methods

2.2.1. Sample collection

Because of its high genetic variability, the ORF5 gene was selected for analyses. A collection of 1127 ORF5 sequences of type 1 PRRSV were prepared and divided into two subsets. The first subset (n = 193) contained sequences originated from Korea with a known collection date and sampling location. The 193 sequences were either generated in this study or retrieved from GenBank and covered a sampling period from 2005 to 2013. These data were used to reconstruct the evolutionary history and epidemiological dynamics of type 1 PRRSV circulating in Korea. Sequences from the first subset were screened for recombination by RDP, GENECONV, and MaxChi methods implemented in the RDP3 program (Martin et al., 2010). None of detection methods identified recombination signals (data not shown). The second subset (n = 934) was downloaded from GenBank regardless of collection date and contained sequences from Korea (n = 11) and other countries (n = 923). This subset was used to precisely classify Korean type 1 PRRSV. The details of these datasets are summarized in supplementary Table S2.1.

2.2.2. Phylogenetic inference

Based on a dataset of 133 ORF5 sequences (originated from Korea and from other countries), an early molecular analysis of type 1 PRRSV isolated in Korea

suggested that Korean type 1 PRRSVs formed three distinct clusters (I, II, and III) from other European genotype PRRSV strains (Kim et al., 2010). Because increased sampling of taxa is one of the most important means to increase overall phylogenetic accuracy (Zwickl and Hillis, 2002), this study challenged the previous finding by inferring the Korean type 1 PRRSV phylogeny from the large collection of sequences mentioned above. The ORF5-based phylogeny was reconstructed on the basis of the maximum likelihood criterion implemented in the simultaneous alignment and tree estimation SATé 2.2.4 program (Liu et al., 2012) with the following options: 'Aligner' = MAFFT, 'Merger' = OPAL, 'Tree Estimator' = RAxML, 'Model' (of nucleotide evolution) = GTRCAT, and 'Quick set' = SATé-II ML. In addition, the reliability of the division of Korean type 1 PRRSV into different clusters was tested by performing pairwise sequence comparisons under the Clustal W algorithm (MegAlign, DNASTAR Lasergene version 7.0.0). The calculated pair similarities were then plotted on frequency versus identity percentage histograms using Microsoft Excel to examine whether the distributions of percentage identities within clusters were different.

2.2.3. Coalescent-based Bayesian Markov chain Monte Carlo analysis

The coalescent-based Bayesian Markov chain Monte Carlo (MCMC) method (Drummond et al., 2012) was applied under the assumption of a codon-based SRD06 nucleotide substitution model (Shapiro et al., 2006) in combination with (i) two models of uncorrelated exponential and uncorrelated lognormal relaxed

molecular clock models and (ii) six demographic coalescent models (constant population size, expansion, exponential, logistic population growth, Bayesian skyline plot, and Bayesian skyride). In each analysis, four independent runs (50 million chains, sampling every 5,000 generations) were performed using the BEAST package v1.7.4 (Drummond et al., 2012), which is available at the Bioportal server (Kumar et al., 2009). The output log files from multiple runs were combined (with proper burn-in steps) using LogCombiner v1.6.2. The combined log files were subsequently analyzed in Tracer v1.5 to assess the convergence (effective sample size > 100) and calculate Bayes factors (BF) for selection of best-fit data models of molecular clock and coalescent tree priors. The interpretation of BF follows previous guidelines (Kass and Raftery, 1995): 2>2 $\log_e$(BF)>0, 6>2 $\log_e$(BF)>2, 10>2 $\log_e$(BF)>6, and 2 $\log_e$(BF)>10 indicate no, positive, strong, and very strong evidence against the null model.

2.2.4. Estimating the absolute rates of synonymous and non-synonymous substitutions

The estimation of the absolute synonymous E[S] and non-synonymous E[N] substitutions rates of the ORF5 gene was performed by a codon-based extension of the Bayesian relaxed clock model (Lemey et al., 2007). The overall procedure can be summarized in the following steps: (i) the Bayesian MCMC method as implemented in BEAST 1.7.4 (Drummond et al., 2012) was used to obtain a subset of posterior distribution trees under the data best-fit models of the relaxed

molecular clock and coalescent models. (ii) Branch lengths in their synonymous and non-synonymous components were decomposed by maximum likelihood estimation using HyPhy 2.1.2 (Pond et al., 2005) based on 200 trees sampled from the posterior distribution. (iii) Finally, the E[S] and E[N] rates for all, external, and internal branches were obtained using the JavaScript code RateAnalyzer.jar (Lemey et al., 2007).

2.2.5. Inferring site-to-site substitution patterns of the ORF5 gene

The synonymous E[S|i] and non-synonymous E[N|i] substitution rates at site i^{th} of the ORF5 gene were estimated by the dual variable rates model (Pond and Muse, 2005) implemented in the dNdSRateAnalysis.bf batch file, HyPhy 2.1.2 (Pond et al., 2005). In all analyses, we used the independent general discrete distribution with three bins for synonymous and non-synonymous rates, and the recommended rate matrix MG94xREV. The input phylogenetic tree was inferred using PhyML 3.0 (Guindon et al., 2010), under assumptions of (i) HKY85+I+Γ4 for model of nucleotides substitution, and (ii) subtree pruning and regrafting topological moves for searching the tree space.

2.2.6. Identifying positively selected site of the ORF5 gene

For diversifying selection inference at sites, this study applied five algorithms of SLAC, FEL, IFEL, MEME, and FUBAR implemented at the Datamonkey web server of HyPhy package (Delport et al., 2010). Because the server excluded

duplicate sequences, 138 and 40 ORF5 sequences of cluster I and cluster II, respectively, were left to the analyses. Sites were inferred to be positively selected at a p-value $\leq$ 0.05 for the SLAC, FEL, IFEL, and MEME methods or by possession of a posterior probability $\geq$ 95% for the FUBAR method.

2.2.7. Inferring epidemiological dynamics of type 1 PRRSV

The epidemic histories of clusters I and II of Korean type 1 PRRSV were determined by reconstructing past population dynamics and estimating the effective reproductive number. Supported by BF tests, the Bayesian skyride outperformed the others coalescent models. The demographic histories of clusters I and II were inferred from the Bayesian skyride model utilizing Tracer v1.5. To model transmission and calculate the effective reproductive number (R, the number of expected secondary infections of an infected individual) of type 1 PRRSV in Korea, the Bayesian birth-death skyline plot method (Stadler et al., 2013), as implemented in the BEAST2 software framework (http://beast2.cs.auckland.ac.nz), was applied. The Bayesian birth-death skyline plot method models epidemiological transmission as a birth-death process and relaxes the assumptions of constant transmission rate, constant noninfectious rate, and constant sampling probability by allowing these parameters to change in a piecewise, constant fashion. The other assumptions for Bayesian MCMC inference were the SRD06 model for sequence evolution (Shapiro et al., 2006) and the exponentially distributed, uncorrelated relaxed clock model.

2.3. Results

2.3.1. ORF5-based phylogeny of type 1 PRRSV in Korea

The phylogenetic relationships of Korean type 1 PRRSVs were investigated in a broad context of the virus circulating worldwide. Depicted in Figure 2.1, type 1 PRRSVs in Korea were grouped within pan-European subtype 1, but belonged to distant branches of the phylogeny. They were previously assigned as clusters I, II and III. No sequences other than those of Korean origin were found on the branches leading to Korean type 1 PRRSVs. This result suggested local diversifying evolution of type 1 PRRSV in Korea since it was first introduced. Of the three clusters, cluster I was the most commonly identified in all provinces (154/204 sequences). The consecutive detections from 2005- 2013 indicated the persistence and successive transmission chains of this cluster among pig farms. To a lesser extent, cluster II was only detected in certain areas, represented by small numbers of sequences (49/204), the majority of which were recovered in 2010 and 2012. By contrast, cluster III was rare (only one sequence was found in 2009) and seemed to no longer exist. Regarding ORF5 nucleotide diversity, the result of pairwise sequence comparisons indicated that the most prevailing clusters I and II exhibited 80.1% - 86.6% sequence similarities and displayed a clear bimodal distribution of nucleotide identities (inserted histogram, Figure 2.1). Regarding phylogeny temporal structure, although cluster I displayed admixture of sequences

collected from different time points, cluster II showed a clear separation of sequences isolated in years 2010 and 2012 from those isolated earlier.

2.3.2. ORF5-based evolutionary dynamics of type 1 PRRSV in Korea

In this section, the evolutionary dynamics of clusters I and II were uncovered by quantifying (i) the nucleotide substitution rates; (ii) the absolute rate of synonymous E[S] and non-synonymous E[N] substitutions, including summaries of all branches, the external branches, and the internal branches of the viral phylogenies; (iii) the site-to-site rate patterns of the entire ORF5 gene; and (iv) the selective pressures acted on each site of each cluster.

2.3.2.1. Evolutionary rates

Under the best-fit molecular clock of uncorrelated, exponential distribution and Bayesian skyline plot demographic models (based on a Bayes factors test), the coefficient of variation (CoV) of the branch rates and the 95% highest posterior density (HPD) intervals were always >0 (CoV = 0.84, 95% HPD: 0.75 to 0.93 for cluster I, and CoV = 0.96, 95% HPD: 0.81 to 1.12 for cluster II). These results indicated large-scale branch rate heterogeneity which suggested non-clock-like behavior. The geometric mean evolutionary rates for cluster II (1.05×10^{-2} substitutions/site/year) were 1.21-fold higher than those for cluster I (0.87×10^{-2} substitutions/site/year), but the 95% HPD intervals overlapped (0.67×10^{-2} to 1.09×10^{-2} for cluster I and 0.75×10^{-2} to 1.44×10^{-2} for cluster II).

2.3.2.2. Absolute rates of synonymous and non-synonymous substitutions

The nucleotide substitution rates were decomposed into the absolute rates of synonymous (E[S]) and non-synonymous (E[N]) substitutions to assess the contributions of each component to the overall nucleotide substitutions. As depicted in Figure 2.2A, the E[S] rates estimated for all branches, the external branches, and the internal branches were similar between clusters I and II. In contrast, Figure 2.2B showed that, although the magnitude of differences in E[N] rates between the clusters was small (estimated for all branches, cluster II substituted at 1.47- fold higher than cluster I), the result was remarkable because the 95% HPD intervals partially overlapped, and the intervals of cluster II excluded the mean rate of cluster I. Similarly, a significance of 1.69-fold difference in E[N] rates, which was estimated solely for the external branches, was observed between the clusters. However, the E[N] rates inferred for internal branches did not differ significantly between the clusters.

2.3.2.3. Site-to-site rate patterns

The expected posterior rates across the ORF5 gene for site i^{th} (E[S|i] for synonymous rates and E[N|i] for non-synonymous rates) were summarized in Figure 2.3. As observed in both clusters I and II (Figure 2.3A, 2.3C), not only E[N|i] but also E[S|i] showed substantial variation. Considered sites with a higher E[N|i] than E[S|i] which were treated as potentially under positive selection, cluster II had more sites (n = 49) than those of cluster I (n = 23). In cluster I

(Figure 2.3B), these sites tended to be distributed in the regions of the signal peptide, ectodomain 1 and 2. In cluster II (Figure 2.3D), they were dispersed across the entire gene rather than limited to the above-mentioned regions.

2.3.2.4. Diversifying selection exerted on ORF5 gene

In this section, several lines of evidence for the unequal intensity of diversifying selection between cluster I and cluster II of Korean type 1 PRRSV are presented. First, cluster I was found to have an elevated E[N]/E[S] ratio (K) relative to cluster II on the internal compared to the external branches: K_{Int}/K_{Ext} = 1.13, 0.66, respectively (data table, Figure 2.2). According to the previous study (Pybus et al., 2007), the results indicated a high-frequency non-synonymous changes on the internal branches of cluster I and an excess of recent non-synonymous changes on the external branches of cluster II. In the next step, the five methods of SLAC, FEL, IFEL, MEME, and FUBAR were separately implemented on the ORF5 alignment of clusters I and II. Each method differently predicted a set of sites to be positively selected (not shown). Still, only sites that were consistently predicted by all of the applied methods were considered; these are reported in Table 2.1. For cluster I, 12 sites were suggested to undergo positive selection out of the 23 sites of potentially candidate for positive selection. Surprisingly, for cluster II, out of the 49 sites with E[N|i] > E[S|i] only site 36 was positively selected with statistical significance. Most of positively selected sites (Table 2.1) were located in the signal peptide, while few sites resided on the ectodomains, and a single site was found on the endodomain of the GP5 protein.

2.3.3. ORF5-based epidemiological dynamics of type 1 PRRSV in Korea

2.3.3.1. Past population dynamics

The best-fit data model indicated that the times of the most recent common ancestor for cluster I and cluster II were approximately 2004 and 2006, respectively. The reconstruction of Bayesian skyride plots suggested that cluster I (Figure 2.4A) had a much larger effective population size than cluster II (Figure 2.4B), and each cluster displayed a different type of population growth dynamics. Although cluster II largely maintained an almost constant population size, cluster I immediately went through an increase in population size, which was maintained for the period from 2004 – 2010, and then declined.

2.3.3.2. Temporal changes of epidemic spread

The birth-death skyline plot revealed a significant difference in the temporal changes of epidemic spread between clusters I and II of Korean type 1 PRRSV. For cluster I (Fig. 2.5A), prior to 2008, the R value was larger than 1, indicating a growing epidemic. During that period, the birth-death skyline plot additionally showed that the epidemic growth peaked twice. Since 2008, the $R \leq 1$, which held until recently, was observed for this cluster. For cluster II (Fig. 2.5B), the opposite epidemiological dynamics were observed. Prior to 2008, the R value estimated for this cluster was slightly above 1; this time was followed by a period in which R increased, then declined until 2011, seeming to increase continuingly thereafter.

2.4. Discussions

On the basis of phylogenetic analyses, circulating type 1 PRRSV in Korea was suggested to have evolved independently on a nationwide scale (Lee et al., 2010), forming distinct clusters from other European genotype PRRSV strains (Kim et al., 2010). These conclusions were drawn from a small set of ORF5 sequences. With the wealth of available ORF5 sequences in GenBank, the phylogenetic classification of PRRSV should be revisited. The first part of this study reconstructed the phylogenetic relationships of Korean type 1 PRRSV from a large, worldwide collection of ORF5 sequences. The hope was that by using a larger dataset, loosely related sequences would segregate into different branches, allowing higher resolution of branching patterns. With this approach, our study confirmed the previous classification of Korean type 1 PRRSVs into three unique clusters. Based on a long-term collection (2005 – 2013) of ORF5 sequences, our results suggested no further introduction of any genetically novel type 1 PRRSV into Korean pig farms and, to date, that only two clusters actually circulate. Furthermore, field isolates belonging to each of the two prevailing clusters were found to group tightly. Several lineages of type 1 PRRSV that emerged in some countries, such as UK, USA, and Thailand, also displayed a tight correlation of the tip character (country) with phylogeny (not shown). This result implies that the local diversifying evolution of type 1 PRRSV upon introduction to a pig population, at country level, might be common worldwide.

The ORF5-based phylogenetic analysis indicated that clusters I and II of the Korean type 1 PRRSV were genetically unrelated and might have undergone unique evolutionary trajectories. Using the estimated nucleotide evolutionary rates, the evolutionary dynamics between clusters I and II were almost obscured due to small difference in their evolutionary rates (the 95% HPD intervals largely overlapped, suggesting these rates were not drawn from different distributions). With the application of a codon-based extension of the Bayesian relaxed clock model (Lemey et al., 2007), this study was able to distinguish between synonymous and non-synonymous substitutions to demonstrate two important attributes. First, the synonymous substation rate, which is related to replication frequency (Hanada et al., 2004), was found to be similar between clusters I and II (Figure 2.2A). This finding implies little difference in the rates of replication between the genetically distinct viruses of Korean type 1 PRRSV. Secondly, the non-synonymous substitution rate averaged on all branches of the viral phylogeny differed significantly between clusters I and II, and the substitution loaded on external branches mostly corresponded to that dissimilarity (Figure 2.2B).

The ORF5-based evolutionary dynamics of Korean type 1 PRRSV were further addressed by characterizing and comparing selective pressures among sites between cluster I and cluster II. Consistent with the higher E[N] rates measured for all branches (Figure 2.2B), the dual variable rates model predicted more than twice the numbers of site potentially subject to positive selection in cluster II (n = 49) than in cluster I (n=23). However, most of the sites of cluster II were not

predicted to undergo diversifying selection (Table 2.1). From the analyses of the absolute synonymous and non-synonymous substitution rates, that cluster had a higher E[N]/E[S] ratio of external branches than of internal branches. According to the previous study (Pybus et al., 2007), the result suggests that many of the non-synonymous sites seen in cluster II were deleterious or slightly deleterious mutations that could not achieve a high rate of fixation. In a sharp contrast, cluster I had a higher E[N]/E[S] ratio of internal branches than of external branches, indicating an unusually strong or recurrent positive selection (Pybus et al., 2007). Collectively, those results explain well why fewer sites were predicted under diversifying selection in cluster II than in cluster I.

Different regions of the GP5 protein were predicted under positive selection pressure, and the present results generally agreed with the findings of previous studies (Delisle et al., 2012, Hanada et al., 2005). Most of the positively selected sites of cluster I were located in the signal peptide, while two sites (positions 104 and 105) were found within the putative ectodomain 2. Nevertheless, the roles of these regions were not experimentally demonstrated for PRRSV; thus, it was impossible to infer their impact. Two positively selected sites 35 and 36 of cluster I and cluster II, respectively, were found in the linear epitopes of the ectodomain (amino acids 29-35 (Wissink et al., 2003), 38-54 (Plagemann, 2004)). We thus hypothesize that these sites of the Korean type 1 PRRSVs might be involved in the adaptation process under immune selection pressure. Site 174 was also observed to be under positive selection and within the immunodominant peptide,

spanning residues 170-201 (Rodriguez et al., 2001). However, this site belongs to the endodomain of the GP5 protein and might not be exposed on the viral surface. Thus, selective pressure, others rather than those from the immune system, could have been exerted on this site.

The last part of this study provided insights into the epidemiological dynamics of type 1 PRRSV in Korea by inferring past population dynamics and temporal changes of the effective reproductive number (R), a key epidemiological parameter. Despite the differences in the underlying frameworks modeling the phylogenetic tree in the Bayesian skyride method (coalescent process) and in the Bayesian birth-death skyline plot method (birth-death process), the generated results (paired Figure 2.4A, 2.5A, and Figure 2.4B, 2.5B) generally correlated. Accordingly, the increasing or decreasing/stabilizing period of the effective population size of both clusters coincided with the prediction of the epidemic growth period ($R > 1$) or epidemic decline/stability period ($R < 1$ or slightly higher than 1). However, the Bayesian birth-death skyline plot revealed more details regarding transmission waves of the endemic caused by Korean type 1 PRRSV. Notably, the fluctuation of the epidemic spread of cluster II which was almost hidden on the basis of the Bayesian skyride inference was better uncovered throughout the Bayesian birth-death skyline plot.

The observation of $R \leq 1$ since 2008 for cluster I (Fig. 2.5A), in theory, seems to indicate that the epidemic is dying out. Nevertheless, PRRSV can persistently infect an individual (Wills et al., 2003) or a population level (Chand et al., 2012).

Further, our sampling data showed that cluster I were more commonly identified (154/204 samples) and was consecutively detected from 2005 to 2013. Thus, a more recent declining epidemic (not fade-out) is a more appropriate inference for the epidemiological dynamics of cluster I. For cluster II, the virus was found only in certain areas, in contrast to the widely distributed of cluster I. The limited distribution of cluster II potentially created a boundary for the transmission of the virus to other herds to expand its population size. The epidemic growth seen recently for this cluster (Fig. 2.5B) might due to an increasing fraction of the susceptible population in the areas where cluster II emerged. In experimental studies, pathogenicity has been demonstrated to influence the transmission of PRRSV (Cho et al., 2007), and highly pathogenic PRRSV is known to replicate at a higher titre than the other typical viruses of the same genotype (Guo et al., 2013, Karniychuk et al., 2010) and is rapidly transmitted to contacted animals (Guo et al., 2013). Thus, another reason for the observed difference in epidemic growth between the two clusters was likely a result of differences in pathogenicity. Unfortunately, there is no comparative information regarding the pathogenicities of cluster I and cluster II, although the pathogenicity of cluster I has been experimentally confirmed (Kim et al., 2011a). From the ORF5- based analyses of evolutionary dynamics, our results suggested that cluster I was more adaptive than cluster II, perhaps contributing to the more successful dispersion of cluster I over pig populations in Korea than that of cluster II did. However, these results should be interpreted with caution because there is increasing evidence for the

contribution of multigenic factors to the biological properties (virulence, antigenicity, etc.) of PRRSV (Allende et al., 2000, An et al., 2011, Leng et al., 2012c). Because cluster I is predominant in Korean pig farms, the recent trends of decreasing effective population size and epidemic growth could be inferred for type 1 PRRSV that has emerged in Korea.

In conclusion, the ORF5-based analyses provided up-to-date information on the prevalence of type 1 PRRSV in Korea that strongly suggested that no further introduction of any genetically novel type 1 PRRSV into Korean pig farms has occurred and that only two clusters actually circulate currently. The evolutionary dynamics was uncovered to be unique between clusters I and II in the terms absolute substitution rates and differential selection pressure exerted on the ORF5 gene. Finally, this study revealed that each cluster of Korean type 1 PRRSV experienced a unique epidemic growth and that there are correlations between the effective population size and the effective reproductive number.

Table 2.1. List of sites predicted to be positively selected in the ORF5 gene of clusters I and II

Phylogenetic cluster	Site[a]	Region of ORF5[b]	SLAC dN-dS[c]	FEL dN-dS	IFEL dN-dS	MEME[d]		FUBAR[e]
						β^+	Pr[β^+]	β-α
I	5	SP	3.84	0.52	0.68	1.45	1.00	0.30
I	8	SP	3.13	0.47	0.93	1.23	1.00	0.26
I	10	SP	3.56	0.54	0.45	1.51	1.00	0.31
I	11	SP	3.75	0.53	0.89	1.39	1.00	0.30
I	12	SP	2.24	0.28	0.39	0.98	0.82	0.22
I	20	SP	6.19	0.91	0.69	2.51	1.00	0.82
I	22	SP	2.45	0.32	0.35	0.94	0.93	0.22
I	25	SP	4.56	0.51	0.44	1.42	1.00	0.32
I	35	EcD1	2.99	0.37	0.48	1.07	1.00	0.26
I	104	EcD2	6.37	1.51	1.81	13.32	0.45	2.01
I	105	EcD2	9.23	1.91	2.31	5.67	1.00	2.12
I	174	EnD	7.22	1.51	1.49	5.66	0.89	2.03
II	36	EcD1	18.48	60.91	90.97	104.93	0.32	5.59

(a): only sites consistently predicted to be positively selected over the different methods were listed. All sites reported in this table were significant at p-value ≤ 0.05 for the SLAC, FEL, IFEL, and MEME methods or had a posterior probability ≥ 95% for the FUBAR method.

(b): signal peptide (SP), putative ectodomain 1 (EcD1), putative ectodomain 2 (EcD2) and endodomain (EnD) of type 1 PRRSV according to the previous study (Stadejek et al., 2002).

(c): normalized synonymous substitution rate (dS) and non-synonymous substitution rate (dN) at a site.

(d): lineage-specific unrestricted non-synonymous rate estimate for the indicated site (β^+) and proportion of branches estimated to be a part of this rate class ($Pr[\beta^+]$).

(e): posterior distribution of non-synonymous substitution rates (β) over sites and posterior distribution of synonymous substitution rates (α) over sites.

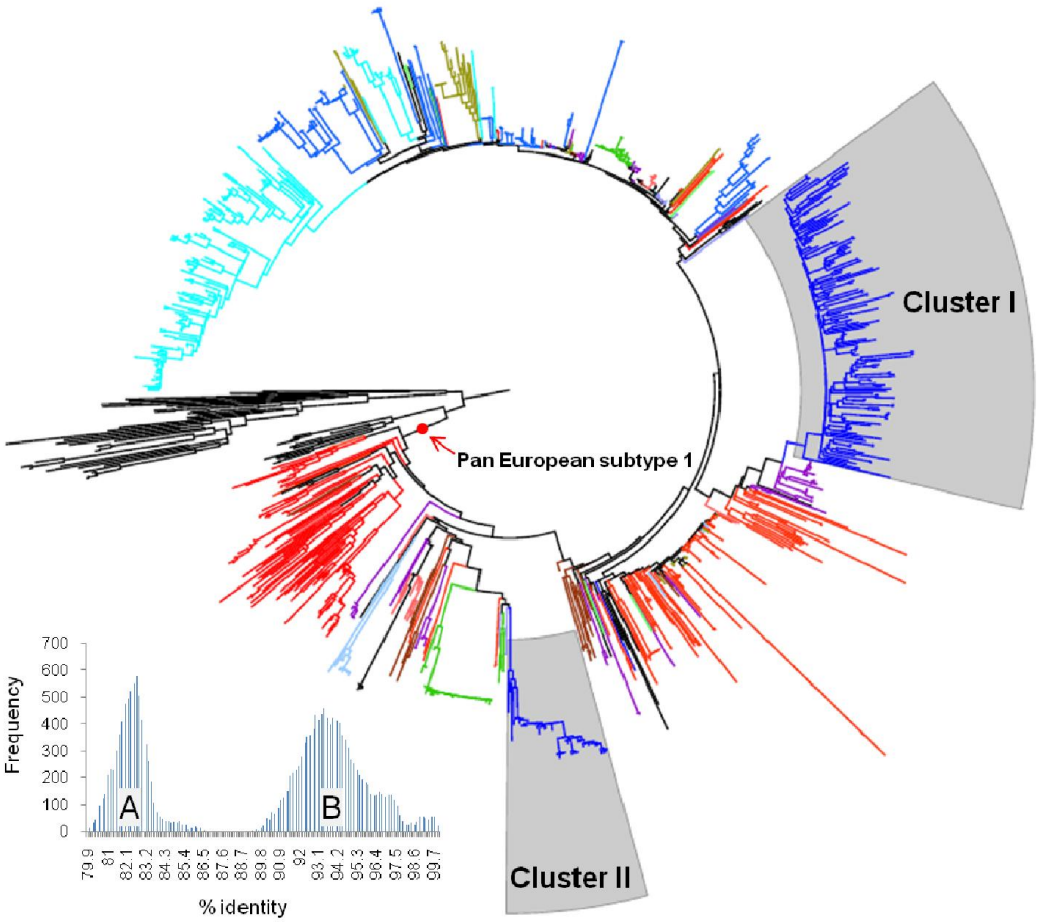
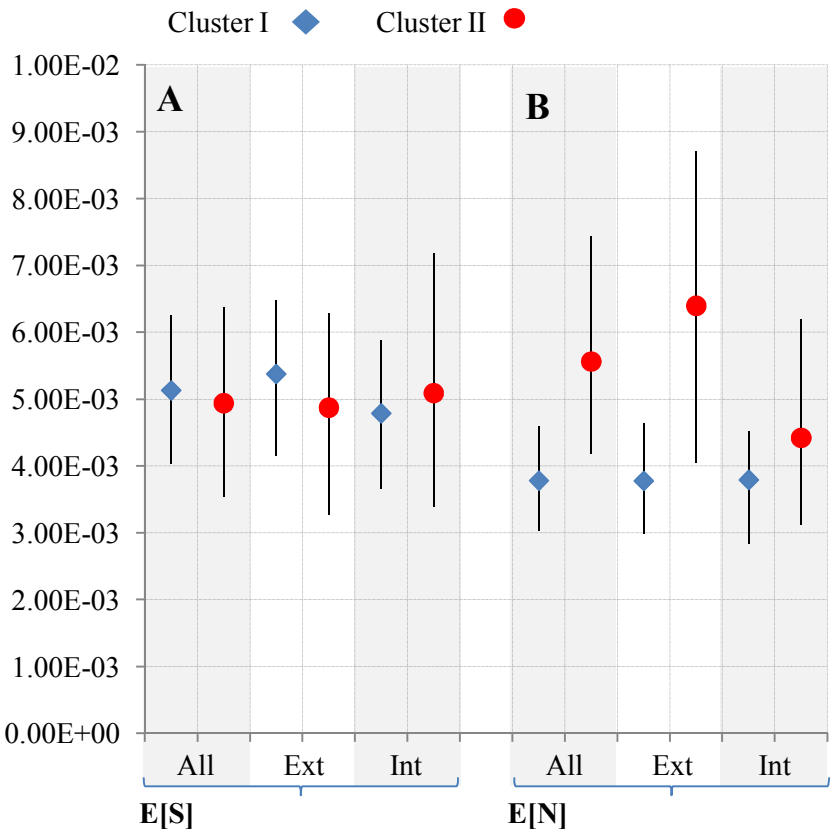


Figure 2.1. ORF5-based maximum likelihood phylogeny of worldwide type 1 PRRSV. Branches of pan-European subtype 1 were colored according to the country of origin. The two major prevailing type 1 PRRSVs in Korea (clusters I and II) are shaded. The inserted histogram depicts pairwise sequence comparisons of Korean type 1 PRRSV, which demonstrated a clear, bimodal distribution of nucleotide identities. The levels of identity between clusters (A) were 80.1% - 86.6%, and within each cluster (B) were 88.9% - 100%.



Cluster	Branch	E[S] x 10^{-3}	E[N] x 10^{-3}	K = E[N]/E[S]	K_{Int}/K_{Ext}
I	Ext	5.38	3.78	0.70	1.13
	Int	4.79	3.79	0.79	
II	Ext	4.87	6.40	1.31	0.66
	Int	5.09	4.42	0.87	

Figure 2.2. Absolute rates of synonymous (E[S]), non-synonymous (E[N]) substitution of the ORF5 gene. (A) and (B) showed comparisons of E[S] and E[N] between cluster I and cluster II over different sets of all, external (Ext), and internal (Int) branches. The data table displayed mean E[S] and E[N] values, the calculations of E[N]/E[S] (K) and the ratio of K between the internal and external branches (K_{Int}/K_{Ext}).

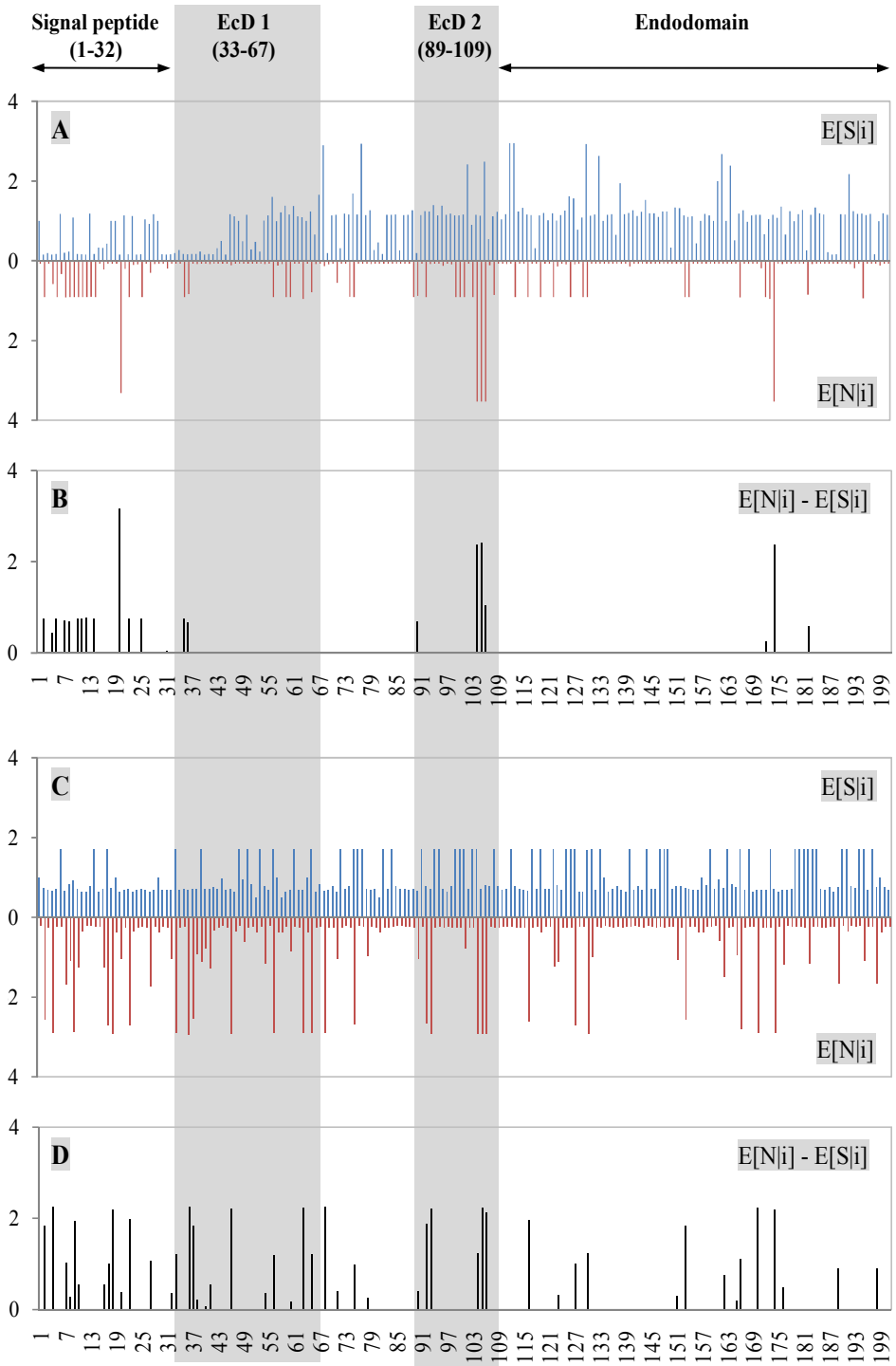

Signal peptide (1-32)
EcD 1 (33-67)
EcD 2 (89-109)
Endodomain
A
E[S|i]
E[N|i]
B
E[N|i] - E[S|i]
C
E[S|i]
E[N|i]
D
E[N|i] - E[S|i]

Figure 2.3. Site-to-site rate patterns across the ORF5 gene. The expected posterior rates across the gene for the i^{th} site (E[S|i] for synonymous rates and E[N|i] for non-synonymous rates) were summarized for cluster I (A) and cluster II (C). The normalized E[N|i] – E[S|i] values solely for sites having E[N|i] > E[S|i] were plotted for cluster I (B) and cluster II (D). Important domains as described previously (Stadejek et al., 2002) were indicated, including the signal peptide, putative ectodomain 1 (EcD1), putative ectodomain 2 (EcD2) and endodomain.

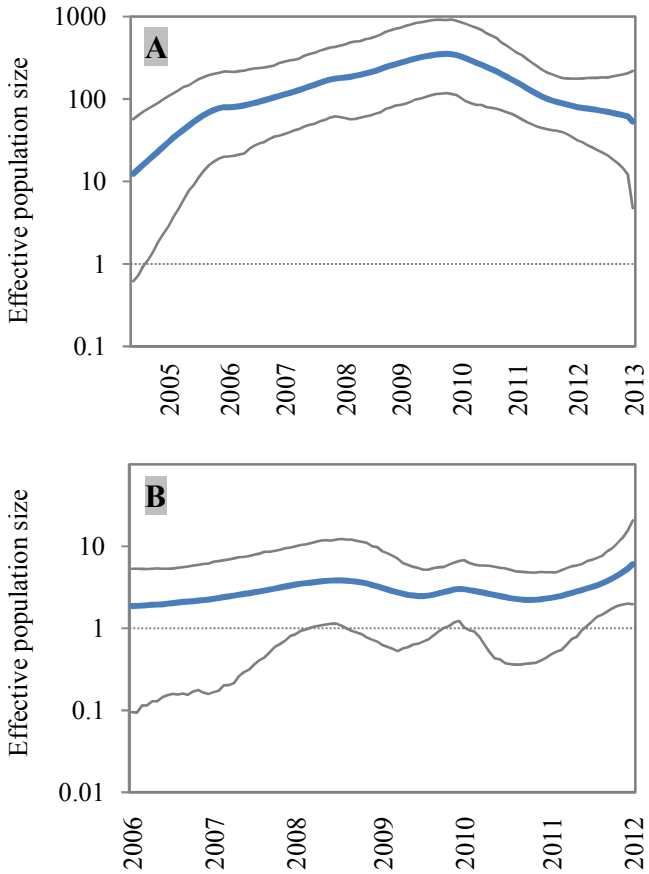


Figure 2.4. ORF5-based demographic histories of Korean type 1 PRRSV were inferred using the Bayesian skyride method for cluster I (A) and cluster II (B). The thick lines represent the estimated effective population size through time. The areas delimited by thin lines represent the 95% highest posterior density intervals.

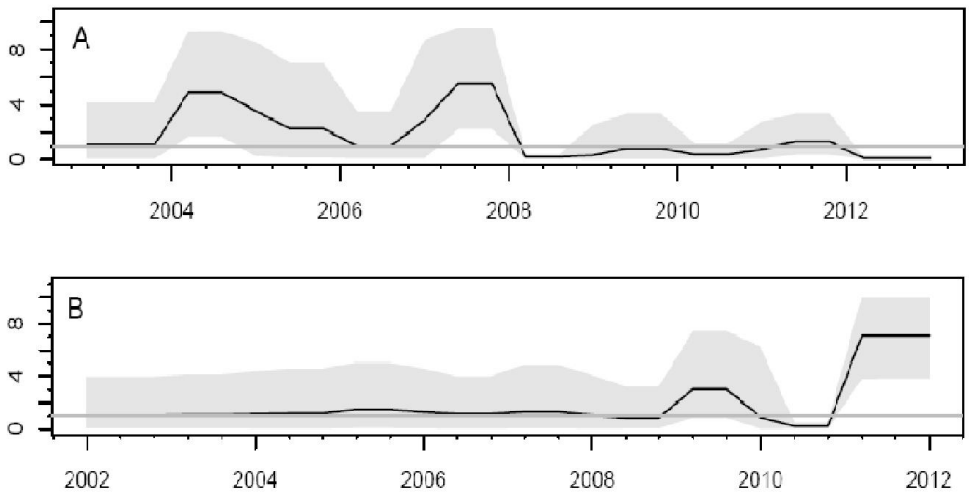


Figure 2.5. ORF5-based birth-death skyline plot of Korean type 1 PRRSV. The birth-death skyline plots depict the fluctuation over time of the effective reproductive number (R, black lines) for cluster I (A) and cluster II (B). The gray areas display the 95% highest posterior density intervals.

Chapter III. Evolutionary dynamics of a highly pathogenic type 2 porcine reproductive and respiratory syndrome virus: analyses of envelope protein-coding genes

Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) has long been an economically devastating swine viral disease. The recent emergence of a highly pathogenic type 2 PRRSV with high mobility and mortality in China, spreading in Vietnam, Laos, and Thailand has placed neighboring countries at risk. This study applied a codon-based extension of the Bayesian relaxed clock model and the fixed effects maximum likelihood method to investigate and compare the evolutionary dynamics of type 2 PRRSV for all of known structural envelope protein-coding genes. By comparing the highly pathogenic type 2 PRRSV clade against the typical type 2 PRRSV clade, this study demonstrated that the highly pathogenic clade evolved at high rates in all of the known structural genes but did not display rapid evolutionary dynamics compared with typical type 2 PRRSV. In contrast, the ORF3, ORF5 and ORF6 genes of the highly pathogenic clade evolved in a qualitatively different manner from the genes of the typical clade. At the population level, several codons of the sequence elements that were involved in viral neutralization, as well as codons that were associated with *in vitro* attenuation/over-attenuation, were predicted to be selected differentially between the typical clade and the highly pathogenic clade. The results of this study suggest that the multigenic factors of the envelope protein-coding genes contribute to diversifying the biological properties (virulence, antigenicity, etc.) of the highly pathogenic clade compared with the typical clade of type 2 PRRSV.

3.1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV), a member of the family *Arteriviridae*, encompasses two genetically and antigenically distinct genotypes (type 1 and type 2) that induce similar disease symptoms of reproductive failure in late gestation sows and respiratory disease in growing pigs. The PRRSV genome is a positive-sense, non-segmented, single-stranded RNA that consists of ten known open reading frames (ORFs). Two large ORF1a and ORF1b genes encode nonstructural proteins that are required for virus replication (Allende et al., 1999). The remaining overlapping genomic regions code for envelope and nucleocapsid proteins in the order 5'-ORF2a-ORF2b-ORF3-ORF4-ORF5a-ORF5-ORF6-ORF7-3' (de Lima et al., 2009, Johnson et al., 2011, Snijder and Meulenberg, 1998, van Nieuwstadt et al., 1996, Wu et al., 2001). All of the envelope proteins are essential for the production of infectious virus (Sun et al., 2013, Wissink et al., 2005); however, the assembly of viral particles is dependent on both the major envelope GP5 and M proteins (Wissink et al., 2005).

Since the discovery of PRRSV, intensive studies of the evolution of the virus at regional as well as global scales have revealed several important traits. The genetic diversity of type 2 PRRSV has increased rapidly over a short period (Shi et al., 2010b), and type 1 PRRSV diverged prior to the first detected outbreak in 1990 in Europe (Stadejek et al., 2002). A striking example of the rapid evolution of PRRSV is the emergence of the highly pathogenic type 2 PRRSV in China, which was the result of local diversification, leading to increased virulence

(Murtaugh et al., 2010, Tian et al., 2007, Zhou et al., 2009a). During the initial identification of the virulence determinants of highly pathogenic type 2 PRRSV, it was believed that a discontinuous deletion of 30 amino-acids in nonstructural protein 2 (nsp2) of type 2 PRRSV corresponded to the shift into a more virulent form (Tian et al., 2007). Later experimental studies suggested that the deletions in nsp2 of highly pathogenic PRRSV did not affect virulence (Zhou et al., 2009b). Paired genomic comparisons of virulent parental/*in vitro* attenuated vaccine viruses of different strains of highly pathogenic type 2 PRRSVs indicated that the determinants of virus attenuation are multigenic products of both the structural and nonstructural genes (An et al., 2011, Leng et al., 2012c). Molecular evolution studies of ORF5 gene have suggested that highly pathogenic type 2 PRRSV is undergoing accelerated evolution (Song et al., 2010) and evolving continuously (Li et al., 2011, Yu et al., 2012). However, extended analysis is still required to elucidate the evolutionary dynamics of the highly pathogenic type 2 PRRSV in the other structural protein-coding genes. Thus, this study set up a comprehensive evolutionary analysis at the population level of all known structural envelope protein-coding genes (ORF2a-ORF6) of type 2 PRRSV. This study aimed to elucidate (i) the evolutionary rates (nucleotide, synonymous, and non-synonymous substitutions) of structural genes and (ii) to dissect which gene(s) and codon(s) might be involved in the differences in evolutionary patterns between typical and highly pathogenic type 2 PRRSV.

3.2. Materials and methods

3.2.1. Sequence data

Genomic sequences of type 2 PRRSV strains were generated in this study (KC771287-KC771288) as well as downloaded from GenBank. Only strains having a complete 3' terminal genome were kept. Type 2 PRRSV was shown to retain a clinical phenotype after a low number of *in vitro* passages and to become attenuated after a high number of serial passages (>19 times) (An et al., 2011, Leng et al., 2012c). Thus, strains with a known number of excessive laboratory passages (>10 times) were also excluded. The final D3 dataset contained 181 sequences. To clarify the phylogenetic relationship, an ORF5-based phylogeny was reconstructed with the maximum likelihood criterion implemented in SATé 2.2.4 (Liu et al., 2012) from a dataset containing (i) 612 reference sequences of lineage 1 to lineage 9 (Shi et al., 2010b) and (ii) the 181 ORF5 sequences compiled herein. As shown in Table S3.1, the type 2 PRRSVs investigated in this study belonged to lineages 1, 2, 3, 4, 5, 8, and 9. With the aim of comparing evolutionary patterns, this study divided type 2 PRRSV into the highly pathogenic clade (D2, n=146), which contained sequences closely clustered with the highly pathogenic type 2 PRRSV that were identified in China (Tian et al., 2007) and its putative common ancestor. The remaining type 2 PRRSV strains were classified as the typical clade (D1, n=35). Each D1-D3 dataset contained seven subsets of each of the ORF2a-ORF6 structural genes.

3.2.2. Bayesian evolutionary analysis

The nucleotide substitution rates of the individual structural gene of type 2 PRRSV were independently estimated from each D1-D3 dataset, using the coalescent-based Bayesian Markov chain Monte Carlo (MCMC) method (Drummond and Rambaut, 2007). The Bayesian MCMC inference was applied under the assumptions of (i) a codon-based SRD06 nucleotide-substitution model (Shapiro et al., 2006), (ii) two models of uncorrelated exponential and uncorrelated lognormal relaxed molecular clocks, and (iii) seven demographic coalescent models (constant population size, expansion growth, exponential growth, logistic growth, Bayesian skyline plot, extended Bayesian skyline plot, and Bayesian skyride). In each analysis, four independent runs (50 million chains, sampling every 5,000 generations) were performed using the BEAST package v1.7.4 (Drummond and Rambaut, 2007), which is available at the Bioportal server (Kumar et al., 2009). The output log files from multiple runs were combined (with proper burn-in steps) using LogCombiner v1.6.2. The combined log files were subsequently analyzed in Tracer v1.5 to assess the convergence (effective sample size > 100) and to calculate the Bayes factors (BF) for the best-fit molecular clock and coalescent tree prior models. The interpretation of the BF follows previous guidelines (Kass and Raftery, 1995): $2>2\ \log_e(BF)>0$, $6>2\ \log_e(BF)>2$, $10>2\ \log_e(BF)>6$, and $2\ \log_e(BF)>10$ and indicate no, positive, strong, and very strong evidence against the null model, respectively.

3.2.3. Estimating absolute rates of synonymous, non-synonymous substitutions

Seven known structural genes of type 2 PRRSV from each of D1-D3 dataset were analyzed with the codon-based extension of the Bayesian relaxed clock model (Lemey et al., 2007) to infer the absolute rates of synonymous (E[S]) and non-synonymous (E[N]) substitutions and to explore how synonymous and non-synonymous divergence had been changing through time. The model decomposes nucleotide substitution rates into synonymous and non-synonymous components on a set of external, internal, and backbone branches from a subset of phylogenetic trees sampled from posterior distributions. Therefore, the model allows for the exploration of the contributions of synonymous and non-synonymous substitutions to the nucleotide substitutions as well as accessing the selective pressure effects on each structural gene. The overall procedure is summarized in the following steps: (i) using the Bayesian MCMC method as implemented in BEAST 1.7.4 (Drummond and Rambaut, 2007) to obtain a subset of posterior distribution trees under the data best-fit models of relaxed molecular clock and coalescent model; (ii) decomposing the branch lengths in their synonymous and non-synonymous components by maximum likelihood estimation using HyPhy 2.1.2 (Pond et al., 2005) based on 200 trees sampled from the posterior distribution; and (iii) summarizing the absolute rates, synonymous and non-synonymous divergence over time for subsets of internal branches using trees in time units and the same set of trees in synonymous/non-synonymous substitution units.

3.2.4. Inferring codon selected differentially between the typical and the highly pathogenic clade

In a population-level phylogeny, deleterious mutations will be young and more likely to fall on the external branches, whereas advantageous mutations are likely to fall deeper in the genealogy and contribute to the persistence of viral lineages through time. Therefore, only internal branches of the type 2 PRRSV phylogeny were tested with the fixed effects maximum likelihood method (Pond et al., 2006) to determine whether selection is operating differentially on the individual codons of a structural gene sampled from the typical clade and the highly pathogenic clade. The method is implemented using the CompareSelectivePressureIVL.bf batch file in HyPhy 2.1.2 (Pond et al., 2005). In all analyses, the default MG94xHKY85 codon model was chosen. The input phylogenetic tree was inferred using PhyML 3.0 (Guindon et al., 2010) under assumptions of (i) the HKY85+I+Γ_4 for model of nucleotides substitution and (ii) subtree pruning and regrafting of topological moves for searching the tree space. Codon p-values$\leq$ 0.05 were considered to be significantly different in terms of the strength of selective pressure between population pairs.

3.3. Results

3.3.1. Rates of evolutionary change

The evolutionary rates of structural genes (ORF2a-ORF6) were inferred from the best-fit relaxed molecular clock and coalescent tree prior models separately for datasets containing only the typical clade (D1), the highly pathogenic clade (D2) of type 2 PRRSV, and the combination of both (D3). In all analyses, the coefficient of variation (Table 3.1) obtained under the relaxed molecular clock was high and indicative of branch rate heterogeneity. Consistently with the D1-D3 datasets, all the investigated genes were estimated to be rapidly evolving on the order of 10^{-3} nucleotide substitutions/site/year (Table 3.1). In addition, the rate heterogeneity among the genes was apparent. The magnitudes of the differences between the highest and the lowest geometric mean rates were 2.31- (D1), 3.25- (D2), and 2.84-fold (D3). Notably, the patterns of evolutionary rates among the structural genes were similar between the typical clade and the highly pathogenic clade (connecting lines, supplementary Figure S3.1). For the same gene, the rates estimated for the typical clade and for the highly pathogenic clade exhibited variations to some extent, with the 95% highest posterior density (HPD) intervals overlapping. Whereas the typical clade had slightly higher rates (1.13 – 1.16-fold higher) in two genes (ORF2a and ORF2b), the virus substituted at lower rates (1.25 – 1.51-fold lower) in other genes (ORF3, ORF4, ORF5a, ORF5, and ORF6) compared to the highly pathogenic clade. Because of the intensive sampling from

the recent epidemic, considerable numbers of transient deleterious mutation segregating on the external branches of the viral phylogeny might not be completely purged by the purifying selection. This, in turn, would artificially increase the substitution rate for the highly pathogenic clade. Eliminating this potential bias, the measurements solely for internal branches indicated that the highly pathogenic clade only substituted at higher rates than the typical clade in the ORF3, ORF4, ORF5a, and ORF6 genes (supplementary Figure S3.1G-H).

3.3.2. Absolute synonymous and non-synonymous substitution rates

The contributions of the synonymous and non-synonymous substitutions to the overall nucleotide substitution were accessed via the absolute rates of synonymous (E[S]) and non-synonymous (E[N]) substitutions. The E[S] and E[N] substitutions rates presented in this section were summarized solely for internal branches. As estimated from each D1-D3 dataset (Figures 3.1A-F), the E[N] substitutions presented remarkable variations among the structural genes compared with the E[S] substitutions, and, therefore, the E[N] substitutions contributed more to the rate heterogeneity among genes. As depicted in Figures 3.1G-H, there was a tendency for faster E[S] substitutions than E[N] substitutions in nearly all of investigated genes, except for the ORF5 and ORF6 genes of the highly pathogenic clade (dashed box, Figure 3.1H), which indicates that the mean E[N] rate was higher than the mean E[S] rate. In the same gene, differences in the mean E[S] and E[N] rate were observed between the typical clade and the highly

pathogenic clade. For synonymous substitution (Figures 3.1A-B), the highly pathogenic clade had substitutions at a rate of 1.43 – 1.48-fold higher than the former clade in the ORF4 and ORF5a genes, whereas the substitution rate was about equal (1.01 – 1.11) and higher rates (1.35 – 1.44) were observed with the typical clade in three genes, ORF2a-ORF3, and the remaining ORF5-ORF6 genes, respectively. For non-synonymous substitution (Figures 3.1D-E), in nearly all genes excluding ORF2a and ORF2b, the highly pathogenic clade displayed higher rates (1.18 – 3.51-fold higher) than in the typical clade.

3.3.3. Comparison of selection pressure

The presence and strength of selection pressure on the envelope protein-coding genes of type 2 PRRSV was evaluated using the E[N]/E[S] ratio. The estimated values (Figure 3.1I) were variable from gene to gene, which indicates unequal selection pressure effects exerted on structural genes within and between the typical clade and the highly pathogenic clade. The ratios obtained for the ORF3, ORF5, and ORF6 genes were always lower in the typical clade than in the highly pathogenic clade, which indicates stronger diversifying selection in the highly pathogenic clade than in the typical clade. In particular, the ORF5 and ORF6 genes were determined to be under positive selection as the E[N]/E[S] ratios were above 1. It is interesting to note that between the two clades, the ORF4 and ORF5a genes were approximately similar in E[N]/E[S] values (dashed circle, Figure 3.1I), implying the same selective pressure might act across those genes.

3.3.4. Changes in the synonymous, non-synonymous divergence through time

To illustrate how synonymous and non-synonymous substitution across structural genes changed over time and whether the accumulation of synonymous and non-synonymous substitutions differed between the typical clade and the highly pathogenic clade, the synonymous and non-synonymous divergences for internal branches were plotted (Figure 3.2). Both of those types of substitutions were observed to be increasing because the number of mutations increases with evolutionary time. The synonymous and non-synonymous substitutions, however, diverged in a non-parallel fashion as the correlation (dashed lines, Figure 3.2) between those components displayed fluctuations through time. For the D1-D3 datasets and almost all structural genes, the synonymous divergence was always larger than the non-synonymous divergence. In a sharp contrast, the ORF5 gene of the highly pathogenic clade exhibited an exceptionally high level of non-synonymous substitutions compared with the level of synonymous substitutions. Comparing the highly pathogenic with the typical clade revealed genes (ORF3 and ORF6) that had accumulated synonymous and non-synonymous substitutions in a qualitatively different manner in which the number of non-synonymous substitutions was higher than the number of synonymous substitutions.

3.3.5. Codons selected differentially between the typical clade and highly pathogenic clade

The maximum likelihood tests evaluating the internal branches of the viral phylogeny identified a number of codons in five out of seven structural genes (except for ORF5a and ORF6) that were selected differentially between the typical clade and the highly pathogenic clade, at $p \leq 0.05$ (Table 3.2). Those codons reside in proteins that (i) are essential for virus replication (GP2a and GP4) (Welch et al., 2004), (ii) are involved in the viral fusion and/or internalization process (E) (Lee and Yoo, 2006), and (iii) are involved in viral neutralization (GP3, GP5) (Cancel-Tirado et al., 2004). Based on the available data in the literature, several of those sites were characterized in detail. For example, codons 69 and 73 (encoded by ORF3) are completely in B-cell linear epitopes (Zhou et al., 2006). Codon 58 (encoded by ORF5) is located in the second hyper-variable region, which is known to be under selective evolutionary pressure (Delisle et al., 2012), as well as to significantly influence the susceptibility of the mutant viruses to a neutralizing antibody (Kim et al., 2013). It was noted that among ten codons predicted to differ in the strength of selective pressure between the typical clade and the highly pathogenic clade, only codon 43 (encoded by ORF4) was demonstrated to occur during the *in vitro* attenuation process of different strains of highly pathogenic type 2 PRRSV (An et al., 2011, Leng et al., 2012c), and codon 172 of that gene was suggested to contribute to the *in vitro* over-attenuation of a highly pathogenic type 2 PRRSV (Yu et al., 2013).

3.4. Discussions

This study investigated seven sets of structural envelope protein-coding genes (ORF2a-ORF6) that were extracted from the type 2 PRRSV complete genome sequence and was able to compare the relative evolutionary rates among the genes. In addition, the collection of genomic sequences was divided into a typical clade and a highly pathogenic clade, which enabled comparisons of the evolutionary dynamics between clades. At the nucleotide level, the structural protein coding genes of type 2 PRRSV generally evolved at approximately 10^{-3} nucleotide substitution/site/year (Table 3.1), which is within the same range as most RNA viruses (Duffy et al., 2008). Although the rate of nucleotide substitution varied with genes, the orders of magnitude was quite narrow (approximately 2.31 – 3.25-fold differences). It is well established that all of the envelope proteins are essential for the production of infectious virus (Sun et al., 2013, Wissink et al., 2005), and some of the envelope proteins are known to interact with each other, such as the disulfide-linked heterodimers of GP5-M proteins (Mardassi et al., 1996) and the non-covalent heterotrimers of GP2-GP3-GP4 proteins (Das et al., 2010, Wissink et al., 2005). In addition, the 3'-region of the PRRSV genome contains overlapping genes encoding structural proteins. As a result, the genome organization and the interactions between the envelope proteins of PRRSV might impose constraints on sequence evolution.

Concerning the evolutionary dynamics, this study demonstrated that the highly pathogenic clade did not rapidly evolve compared with the type 2 PRRSV typical

clade. The maximum nucleotide substitution rate of the highly pathogenic strain was approximately 1.5-fold higher, and in all estimations, the 95% HPD intervals overlapped. In experimental studies, highly pathogenic type 2 PRRSV is known to replicate at a higher titer than the other typical viruses of the same genotype and is rapidly transmitted to contacted animals (Guo et al., 2013). Those properties were expected to increase the nucleotide substitution rates of the highly pathogenic clade over the typical clade, and thus, question remains on the rate of nucleotide substitution determined in this study. However, possible biases (incorrect modeling of sequence evolution and inclusion of transient deleterious mutations) that may skew the estimations were taken into account by inferring the substitution rates from the best-fit molecular clock and demographic models (based on a Bayes factors test) solely for internal branches of the viral phylogeny. As a result, the evolutionary rates determined in this study were validated. In the literature, the difference in molecular evolution between biological scales is suggested to be a common feature of chronic RNA viral infection (Gray et al., 2011). Because PRRSV is known to maintain a life-long subclinical infection and persist at a population level (Chand et al., 2012) and our analyses were based on the population level rather than individual level, the estimated rates might not match the evolutionary dynamics within an individual.

Although rapid evolutionary dynamics were not observed, this study revealed that highly pathogenic type 2 PRRSV evolved in a qualitatively different manner from the typical type 2 PRRSV in the genes encoding the minor envelope protein

(ORF3/GP3), the major envelope protein (ORF5/GP5), as well as the matrix protein (ORF6/M). This evolutionary pattern was not evident in the entire type 2 phylogeny (containing both the typical and the highly pathogenic clades), which was likely due to the selection pressure changes in only the branch leading to the highly pathogenic clade. Therefore, the averaged intensity over all branches might have been diluted. The identification of the operation of selection pressure on important structural genes that were involved in neutralizing and producing infectious particles, among other activities, in at least the three genes mentioned above corresponded to the biological differences between the typical clade and the highly pathogenic clade of type 2 PRRSV. These findings were in line with the previous suggestions regarding the contribution of multigenic factors to the virulence of highly pathogenic type 2 PRRSV (An et al., 2011, Leng et al., 2012c). Further studies of the nonstructural protein-coding genes are warranted to determine the other genetic factors associated with diversifying the biological properties between the typical clade and the highly pathogenic clade.

Another notable result of this study was the inference of codons differentially selected between the typical clade and the highly pathogenic clade at the population level. In combination with the identification of the accumulation of synonymous and non-synonymous substitutions in a qualitatively different manner, the results reflected lineage-specific adaptive responses of the two type 2 PRRSV clades. For example, the GP5 B antigenic region, a major linear neutralizing epitope in PRRSV classical strains, has been shown to not be a

neutralizing antigenic region of highly pathogenic type 2 PRRSV (Leng et al., 2012a), which might be due to mutations in codon 39 of that region of highly pathogenic type 2 PRRSV. On the other hand, codon 58 of ORF5/GP5, which was identified as differentially selected in this study, is located in a region that significantly influences the susceptibility of the mutant viruses to neutralizing antibody (Kim et al., 2013). Thus, our results suggest that codon 58 could affect the GP5 neutralizing epitope. It should be mentioned that none of the glycosylation sites in the GP3 and GP5 envelope proteins that play critical roles in immune evasion (Vu et al., 2011) or are critically important for virus replication *in vivo* (Wei et al., 2012) were differentially selected between the typical and highly pathogenic type 2 PRRSV viruses. These results were likely due to the essential functions of these sites, which are subject to similar and strong selection constraints. Other differentially selected codons (codons 43 and 172 of ORF4/GP4, $p<0.01$) were revealed to be related to the *in vitro* attenuation/over-attenuation processes of highly pathogenic type 2 PRRSV (An et al., 2011, Leng et al., 2012c, Yu et al., 2013). Those sites, however, did not change during the processes of attenuation and reversion to virulence of typical type 2 PRRSV as reported elsewhere (Allende et al., 2000, Grebennikova et al., 2004). In addition, there is no information available about the reversion to virulence of the vaccine virus derived from highly pathogenic type 2 PRRSV. Thus, we cannot draw any conclusions concerning the roles of those differentially selected codons.

In conclusion, it was demonstrated that highly pathogenic type 2 PRRSV evolved at high rates in all of the known structural genes but did not display rapid evolutionary dynamics as compared with typical type 2 PRRSV. In contrast, the highly pathogenic clade was observed to have evolved in a qualitatively different manner from the typical clade of type 2 PRRSV in the ORF3, ORF5 and ORF6 genes. At the population level, several codons of the sequence elements involved in viral neutralization as well as in association with *in vitro* attenuation/over-attenuation were predicted to be differentially selected between the typical clade and the highly pathogenic clade.

Table 3.1. Estimated nucleotide substitution rates of structural protein-coding genes

Gene	Dataset[a]	Mean rate[b] (x 10^{-3})	95% HPD interval (x 10^{-3})	CoV[c]
ORF2a	D1	2.48	1.38 – 3.80	1.52
	D2	**2.13**	**1.63 – 2.67**	0.96
	D3	2.25	1.64 – 2.93	0.97
ORF2b	D1	1.80	0.81 – 3.05	0.96
	D2	**1.59**	**1.02 – 2.31**	0.99
	D3	1.55	1.00 – 2.18	0.99
ORF3	D1	2.28	0.94 – 3.85	1.84
	D2	**2.83**	**2.18 – 3.55**	0.94
	D3	2.52	1.88 – 3.26	0.95
ORF4	D1	2.18	0.70 – 4.13	2.11
	D2	**2.77**	**2.08 – 3.53**	0.98
	D3	2.55	1.79 – 3.51	0.98
ORF5a	D1	3.44	1.55 – 5.91	0.96
	D2	**5.18**	**3.33 – 7.34**	0.99
	D3	4.39	2.99 – 6.10	1.00
ORF5	D1	2.61	0.79 – 4.62	2.05
	D2	**3.33**	**2.47 – 4.34**	0.98
	D3	3.18	2.28 – 4.24	0.99
ORF6	D1	1.49	0.43 – 2.92	2.40
	D2	**2.24**	**1.58 – 3.00**	0.99
	D3	1.63	1.09 – 2.21	0.99

a) D1, D2, and D3 represent the typical clade, the highly pathogenic clade, and the combination of both clades, respectively.

b) The geometric mean nucleotide substitution rate (substitutions/site/year). All of the rates were drawn from the data best-fit molecular clock and demographic models. The estimated rates of the highly pathogenic clade are given in bold face.

c) The coefficient of variation obtained under the relaxed molecular clock

Table 3.2. A list of differentially selected codons in the structural protein-coding genes sampled from the typical clade and the highly pathogenic clade of type 2 PRRSV

Gene	Codon	Typical clade		Highly pathogenic clade		p-value
		dN internal[a]	*dN leaves*[b]	*dN internal*	*dN leaves*	
ORF2a	10	13.15	2.21	0.00	3.92	0.05
	84	4.66	1.78	0.00	4.03	0.03
ORF2b	48	0.00	6.51	16.78	2.83	0.02
ORF3	69	0.00	0.00	3.09	1.75	0.02
	73	0.00	0.86	3.44	0.90	0.01
ORF4	43	4.29	2.63	31.43	14.90	0.00
	154	0.00	0.00	2.84	0.00	0.04
	172	0.00	0.00	6.52	0.00	0.00
ORF5	13	10.10	4.46	0.00	1.64	0.05
	58	11.03	8.64	25.79	9.74	0.03

a,b) Non-synonymous substitution rates along the internal (dN internal) and terminal (dN leaves) branches.

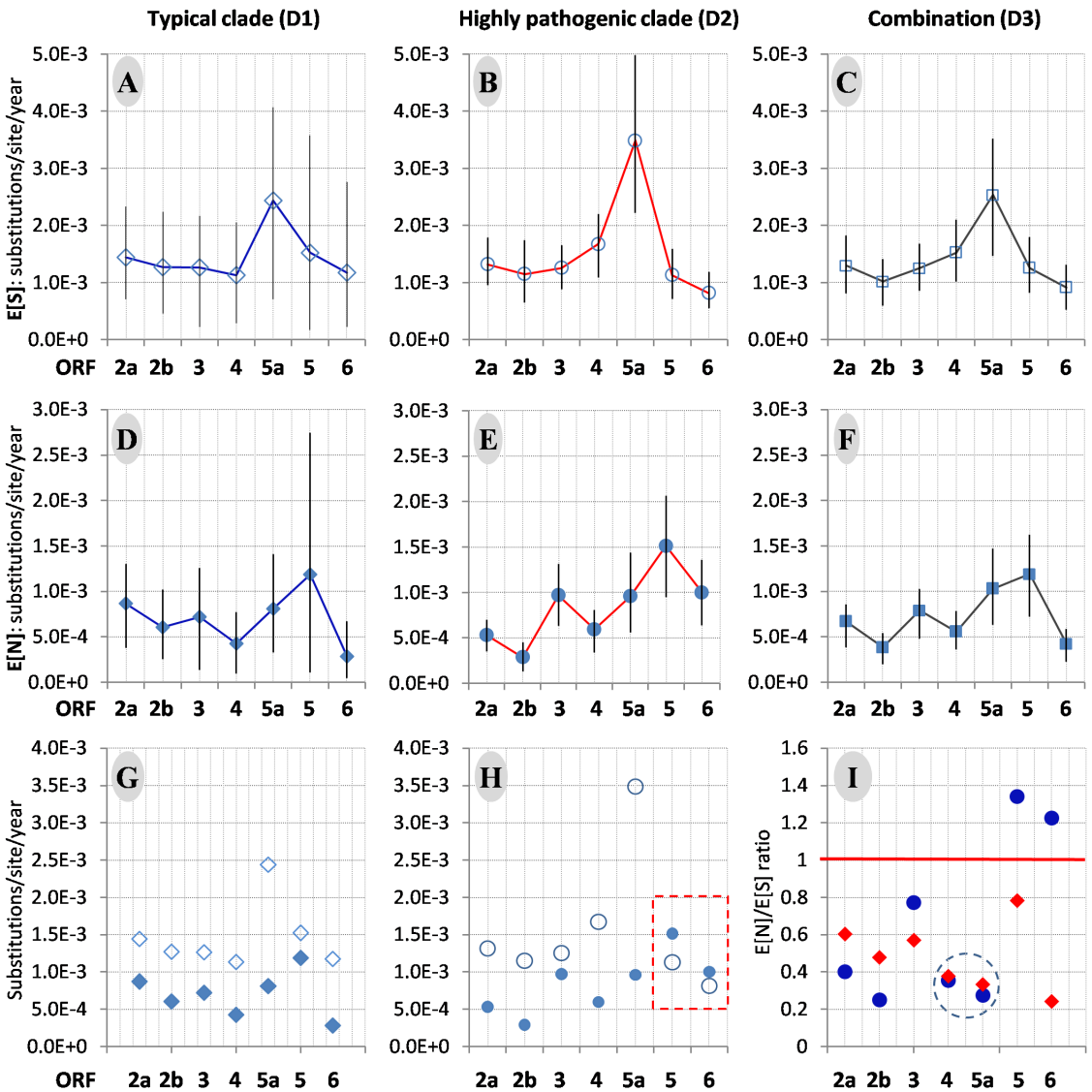


Figure 3.1. Absolute rates of synonymous (E[S]), non-synonymous (E[N]) substitution and 95% highest posterior density interval of the typical clade (A, D), the highly pathogenic clade (B, E), and the combination of both clades (C, F). Comparisons of the E[S] and E[N] for each envelope protein-coding gene of the typical clade (G) and the highly pathogenic clade (H). (I) shows E[N]/E[S] ratios for each gene between the typical clade (diamonds) and the highly pathogenic clade (filled circles).

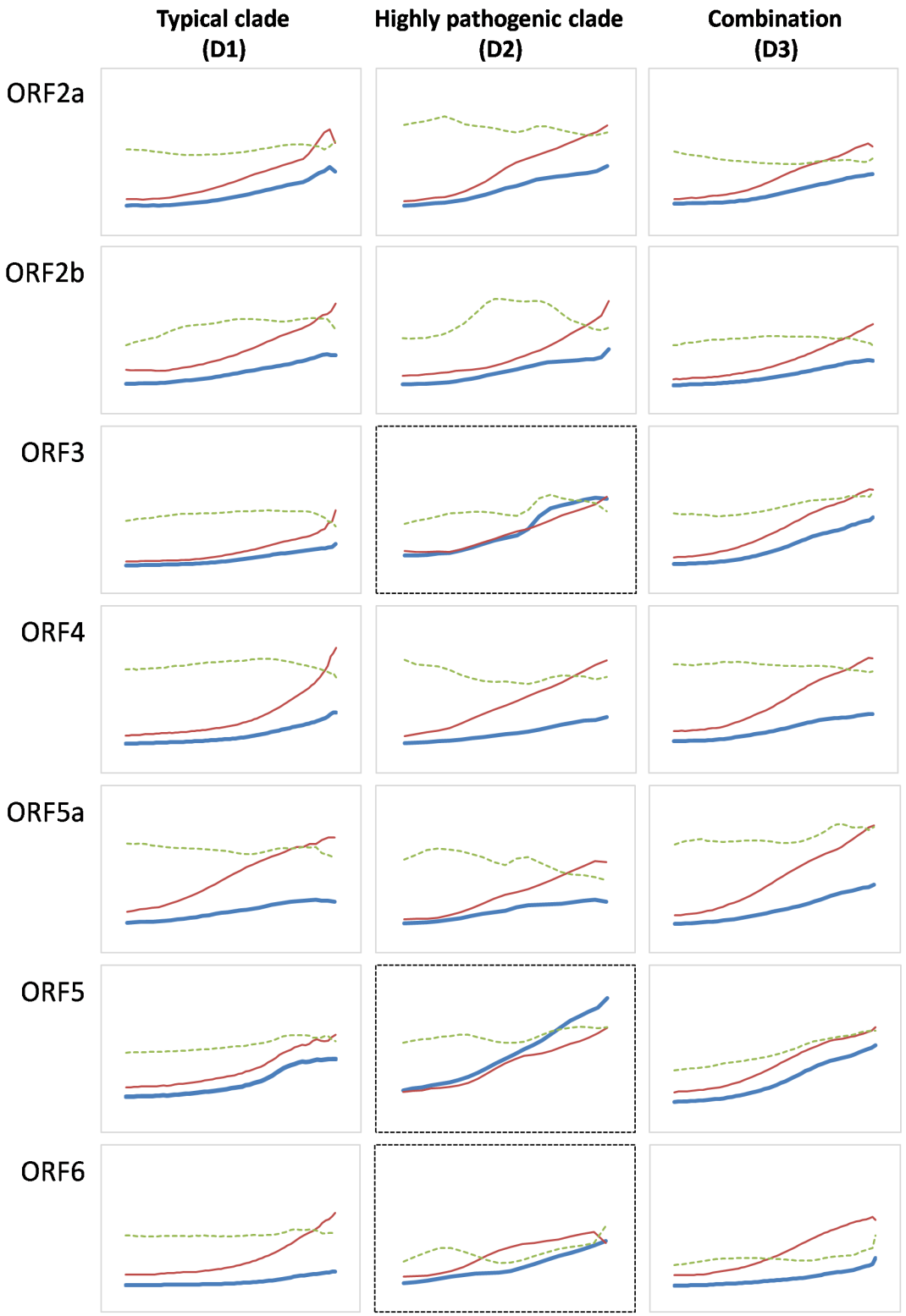

Typical clade
(D1)
Highly pathogenic clade
(D2)
Combination
(D3)
ORF2a
ORF2b
ORF3
ORF4
ORF5a
ORF5
ORF6

Figure 3.2. The synonymous and non-synonymous divergences for each envelope protein-coding gene of the typical clade, the highly pathogenic clade of type 2 PRRSV, and the combination of both clades. Synonymous, non-synonymous divergence and their correlation are shown in thin, thick and dashed lines, respectively. The genes of ORF3, ORF5, and ORF6 of the highly pathogenic clade accumulated synonymous and non-synonymous substitutions in a different manner than the typical clade (dashed boxes).

GENERAL CONCLUSIONS

With the wealth of publicly available genetic sequences of PRRSV in GenBank and the advances in statistical approaches, this study addresses several important aspects regarding the epidemiological and evolutionary dynamics of PRRSV.

The analysis of the epidemiological dynamics of type 1 PRRSV across pig-producing countries suggested that the virus (i) diversified into unique subpopulations for a long time prior to the first recorded endemic and (ii) consecutively diffused among various countries in Europe, indicating that some countries, such as Spain and Germany, acted as distribution sources to a certain extent. Type 1 PRRSV tended to cluster by geographical locations to form distinctive population structures. In each population, the evolution of the ORF5 gene was best described by a non-homogeneous process.

The long-term investigation of the evolutionary trajectories and epidemiological dynamics of type 1 PRRSV in Korea between 2005 and 2013 demonstrated no further introduction of genetically novel type 1 PRRSV into Korean pig farms, with the identification of only two clusters (I and II) in circulation to date. Cluster I was found to have an elevated E[N]/E[S] ratio relative to cluster II on the internal branches compared to the external branches. Far fewer sites were predicted under the diversifying selection in cluster II than in cluster I. Each cluster experienced a unique epidemic growth and identified correlations between effective population size and effective reproductive number.

The genome-scale analyses of evolutionary dynamics, which were performed as an example of the typical clade and the highly pathogenic clade of type 2 PRRSV, showed that the highly pathogenic clade evolved at high rates in all of the known structural genes but did not display rapid evolutionary dynamics compared with typical type 2 PRRSV. In contrast, the ORF3, ORF5 and ORF6 genes of the highly pathogenic clade evolved in a qualitatively different manner from the genes of the typical clade. At the population level, several codons of the sequence elements that were involved in viral neutralization, as well as codons that were associated with *in vitro* attenuation/over-attenuation, were predicted to be selected differentially between the typical clade and the highly pathogenic clade.

Collectively, this study successfully reconstructs the global transmission histories of type 1 PRRSV and uncovers the evolutionary and epidemiological dynamics of the virus at a country level. Extended to the genome-scale analysis of evolutionary dynamics, several structural genes and codons have been found to evolve in qualitatively different manners and were differentially selected between the typical clade and the highly pathogenic clade of type 2 PRRSV.

An intensive investigation of type 1 PRRSV in Korea indicated only two actual circulating clusters, and suggested for the recent trends of decreasing epidemic growth. That yields valuable information in vaccine development, such as viral strain selection for vaccination against type 1 PRRSV in Korea. Future studies should be extended to address the evolutionary dynamics as well as the epidemiological dynamics of Korean type 2 PRRSV. The deep understanding of

the viral evolutionary trajectories is believed useful in the design of more effective disease prevention strategies. In addition, there is increasing evidence for the contribution of multigenic factors to the biological properties of PRRSV. Thus, extending to genome-wide scale analyses in the future might be necessary to study the microevolution of the virus circulating in Korea.

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국문초록

돼지생식기호흡기증후군 바이러스의 역학적 및 유전적 특성 규명

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돼지생식기호흡기증후군 바이러스 (PRRSV)는 Arteriviridae 에 속하고, 유전적 및 혈청학적으로 뚜렷이 구분되는 두 유전자형 (type 1 및 type 2 PRRSV)을 가진다. 지금까지 거의 모든 돼지 생산국에서 본 바이러스가 한 유전자형 또는 두 유전자형 모두 존재하는 것으로 보고되었다. 최근 중국에서 발견된 고병원성 type 2 PRRSV 는 높은 이환율 및 폐사율을 보이는 종으로, 중국과 인접한 국가들이 잠재적 위험에 노출되어 있는 실정이다. PRRSV 의 효과적 예방 및 백신 개발에 있어 병원체의 시공간적 전파 양식과 유전적 다양성 연구가 중요하기에, 본 연구에서는 바이러스의 역학 (epidemiological) 및 진화론적 역학 (evolutionary dynamics)에 관한 연구를 수행하였다.

첫 번째 연구 주제는 1991 년부터 2011 년 사이 세계 각국에서 밝힌 type 1 PRRSV ORF5 염기서열을 이용한 분자역학적 조사로서 Bayesian phylogeographical analysis 기법을 응용하였다. 그 결과 현재 type 1 PRRS 가 이루는 population 들이 지정학적 위치와 긴밀한 관계를 가지며, 각 population 내에서는 non-homogenous evolution 이 가장 많음을 알 수 있었다.

두 번째 연구에서는 범위를 좁혀, 국내 돼지에 감염된 type 1 PRRSV 의 진화에 관해 분석을 시도하였다. Maximum likelihood phylogenetic analysis 를 통해 2005 년부터 2013 년 사이에 밝혀진 ORF5 서열을 분석한 결과, 현재 국내에 존재하는 두 종류의 cluster 이외에 새로운 type 1 PRRSV 의 유입은 없었음을 알 수 있었다. 또한 Bayesian birth-death skyline plot 과 Bayesian relaxed clock model 의 codon-based extension 을 적용하여 다른 cluster 에 속하는 바이러스와 구별되는 국내 type 1 PRRSV 의 특징을 유전적, 역학적 측면에서 밝혔다.

마지막으로 고병원성 type 2 PRRSV 가 가지는 진화론적 역학(evolutionary dynamics)의 특징을 전형적인 type 2 PRRSV 와 비교하였다. 조사 대상 바이러스의 모든 structural envelope protein-coding gene 을 대상으로 Bayesian relaxed clock model 의 codon-based extension 기법 및 fixed effects maximum likelihood 방법을

적용하였다. 실험 결과, 고병원성 바이러스로 구성된 clade 는 진화 속도 측면에서 전형적인 type 2 PRRSV 와 큰 차이를 보이지 않았다. 반면, 고병원성 type 2 PRRSV 의 유전적 변이를 질적인 측면에서 비교해 보았을 때 전형적인 type 2 PRRSV 와는 그 방식이 확연히 다름을 알 수 있었다.

결론적으로, type 1 PRRSV 의 ORF5 유전자를 분석한 연구를 통해, 우리는 본 바이러스의 세계적 전파 역사를 추측할 수 있었고, 국내에 현재 분포하는 바이러스의 진화적 역학에 관한 정보를 얻을 수 있었다. 또한 genome 단위에서 고병원성 PRRSV 를 다른 type 2 PRRSV 와 비교함으로써 다양한 구조유전자 및 codon 이 고병원성 PRRSV 가 현재 가지는 여러 진화적 역학의 특징을 이루는데 기여했음을 알게 되었다. 본 연구를 통해 얻게 된 PRRSV 의 다양한 유전적 정보들은 앞으로 PRRSV 를 방제하기 위한 대책을 수립할 때 유용하게 쓰일 것으로 기대하며 특히 백신 개발을 앞두고 적합한 백신용 strain 을 선별 할 때 유전적 측면에서 올바른 방향을 제시 해 줄 것으로 예상한다.

Key words: 돼지생식기호흡기증후군 바이러스, 분자유전, 역학, 고병원성

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APPENDIX

Supplementary Table S1.1. The details of the ORF5 dataset in analyses in chapter I

No.	*Sequence ID*	*Country*	*Year of isolation*	*Reference*	*No.*	*Sequence ID*	*Country*	*Year of isolation*	*Reference*
1	DQ324669	Belarus	2004	(Stadejek et al., 2006)	194	AY743937	Italy	2003	(Pesente et al., 2006)
2	DQ324686	Belarus	2004	(Stadejek et al., 2006)	195	AY749417	Italy	2003	(Pesente et al., 2006)
3	DQ324690	Belarus	2004	(Stadejek et al., 2006)	196	AY739959	Italy	2004	(Pesente et al., 2006)
4	DQ324694	Belarus	2004	(Stadejek et al., 2006)	197	AY739960	Italy	2004	(Pesente et al., 2006)
5	DQ324676	Belarus	2005	(Stadejek et al., 2006)	198	AY739962	Italy	2004	(Pesente et al., 2006)
6	EU071227	Belarus	2006	(Stadejek et al., 2008)	199	AY739963	Italy	2004	(Pesente et al., 2006)
7	EU071228	Belarus	2006	(Stadejek et al., 2008)	200	AY739964	Italy	2004	(Pesente et al., 2006)
8	JF802085	Belarus	2007	(Van Doorsselaere et al., 2012)	201	AY739971	Italy	2004	(Pesente et al., 2006)
9	AY035900	Belgium	1992	(Forsberg et al., 2002)	202	AY739973	Italy	2004	(Pesente et al., 2006)
10	AY035901	Belgium	1992	(Forsberg et al., 2002)	203	AY739984	Italy	2004	(Pesente et al., 2006)
11	JF304781	Belgium	1994	(Van Breedam et al., 2011)	204	AY739989	Italy	2004	(Pesente et al., 2006)
12	GU047344	China	2006	(Chen et al., 2011)	205	AY739990	Italy	2004	(Pesente et al., 2006)
13	GU047340	China	2009	(Chen et al., 2011)	206	AY740002	Italy	2004	(Pesente et al., 2006)
14	GU047341	China	2009	(Chen et al., 2011)	207	AY743933	Italy	2004	(Pesente et al., 2006)
15	GU047342	China	2009	(Chen et al., 2011)	208	AY743934	Italy	2004	(Pesente et al., 2006)
16	GU047343	China	2009	(Chen et al., 2011)	209	AY743935	Italy	2004	(Pesente et al., 2006)
17	AF253537	Czech	1995	(Indik et al., 2000)	210	EF031037	Korea	2005	Unpublished
18	AF253531	Czech	1996	(Indik et al., 2000)	211	DQ355821	Korea	2006	Unpublished
19	AF253532	Czech	1996	(Indik et al., 2000)	212	EF031038	Korea	2006	Unpublished
20	AF253534	Czech	1998	(Indik et al., 2000)	213	EF031039	Korea	2006	Unpublished
21	AF253535	Czech	1998	(Indik et al., 2000)	214	EF031040	Korea	2006	Unpublished
22	AY035913	Denmark	1992	(Forsberg et al., 2002)	215	EF031041	Korea	2006	Unpublished
23	AY035916	Denmark	1992	(Forsberg et al., 2002)	216	EF031042	Korea	2006	Unpublished
24	AY035944	Denmark	1992	(Forsberg et al., 2002)	217	EF031043	Korea	2006	Unpublished
25	AY035906	Denmark	1993	(Forsberg et al., 2002)	218	EF031044	Korea	2006	Unpublished

No.	*Sequence ID*	*Country*	*Year of isolation*	*Reference*	*No.*	*Sequence ID*	*Country*	*Year of isolation*	*Reference*
26	AY035909	Denmark	1993	(Forsberg et al., 2002)	219	GQ847573	Korea	2006	(Lee et al., 2010)
27	AY035902	Denmark	1995	(Forsberg et al., 2002)	220	FJ349261	Korea	2007	(Nam et al., 2009)
28	AY035903	Denmark	1995	(Forsberg et al., 2002)	221	GU325642	Korea	2007	(Lee et al., 2010)
29	AY035917	Denmark	1995	(Forsberg et al., 2002)	222	GU325643	Korea	2007	(Lee et al., 2010)
30	AY035904	Denmark	1996	(Forsberg et al., 2002)	223	FJ972762	Korea	2008	(Kim et al., 2009)
31	AY035905	Denmark	1996	(Forsberg et al., 2002)	224	FJ972763	Korea	2008	(Kim et al., 2009)
32	AY035907	Denmark	1997	(Forsberg et al., 2002)	225	FJ972764	Korea	2008	(Kim et al., 2009)
33	AY035908	Denmark	1997	(Forsberg et al., 2002)	226	FJ972765	Korea	2008	(Kim et al., 2009)
34	AY035910	Denmark	1997	(Forsberg et al., 2002)	227	FJ972766	Korea	2008	(Kim et al., 2009)
35	AY035911	Denmark	1998	(Forsberg et al., 2002)	228	GQ847574	Korea	2008	(Lee et al., 2010)
36	AY035912	Denmark	1998	(Forsberg et al., 2002)	229	GQ847575	Korea	2008	(Lee et al., 2010)
37	AY035918	France	1991	(Forsberg et al., 2002)	230	GQ847576	Korea	2008	(Lee et al., 2010)
38	AY035919	France	1992	(Forsberg et al., 2002)	231	GQ847577	Korea	2008	(Lee et al., 2010)
39	AY035920	France	1993	(Forsberg et al., 2002)	232	GQ847578	Korea	2008	(Lee et al., 2010)
40	AY035921	Germany	1993	(Forsberg et al., 2002)	233	GQ847579	Korea	2008	(Lee et al., 2010)
41	AY035922	Germany	1993	(Forsberg et al., 2002)	234	GQ847580	Korea	2008	(Lee et al., 2010)
42	AY035923	Germany	1993	(Forsberg et al., 2002)	235	GQ847581	Korea	2008	(Lee et al., 2010)
43	AY035924	Germany	1993	(Forsberg et al., 2002)	236	GQ847582	Korea	2008	(Lee et al., 2010)
44	AY035925	Germany	1993	(Forsberg et al., 2002)	237	GQ847583	Korea	2008	(Lee et al., 2010)
45	JF276434	Germany	1996	(Darwich et al., 2011)	238	GQ847584	Korea	2008	(Lee et al., 2010)
46	GQ451675	Germany	1997	Unpublished	239	GQ847585	Korea	2008	(Lee et al., 2010)
47	FJ705373	Germany	2003	(Greiser-Wilke et al., 2010)	240	GQ847586	Korea	2008	(Lee et al., 2010)
48	FJ705374	Germany	2003	(Greiser-Wilke et al., 2010)	241	GQ847587	Korea	2008	(Lee et al., 2010)
49	FJ705375	Germany	2003	(Greiser-Wilke et al., 2010)	242	GQ847588	Korea	2008	(Lee et al., 2010)
50	FJ705376	Germany	2003	(Greiser-Wilke et al., 2010)	243	GQ847589	Korea	2008	(Lee et al., 2010)
51	FJ705377	Germany	2003	(Greiser-Wilke et al., 2010)	244	GQ847590	Korea	2008	(Lee et al., 2010)
52	FJ705380	Germany	2004	(Greiser-Wilke et al., 2010)	245	GQ847591	Korea	2008	(Lee et al., 2010)
53	FJ705381	Germany	2004	(Greiser-Wilke et al., 2010)	246	GQ847592	Korea	2008	(Lee et al., 2010)
54	FJ705383	Germany	2004	(Greiser-Wilke et al., 2010)	247	GU325644	Korea	2008	(Lee et al., 2010)
55	FJ705384	Germany	2004	(Greiser-Wilke et al., 2010)	248	GU325645	Korea	2008	(Lee et al., 2010)
56	FJ705385	Germany	2004	(Greiser-Wilke et al., 2010)	249	GU325646	Korea	2008	(Lee et al., 2010)
57	FJ705386	Germany	2004	(Greiser-Wilke et al., 2010)	250	GU325647	Korea	2008	(Lee et al., 2010)

No.	*Sequence ID*	*Country*	*Year of isolation*	*Reference*	*No.*	*Sequence ID*	*Country*	*Year of isolation*	*Reference*
58	FJ705391	Germany	2004	(Greiser-Wilke et al., 2010)	251	GU325648	Korea	2008	(Lee et al., 2010)
59	FJ705394	Germany	2004	(Greiser-Wilke et al., 2010)	252	GQ847593	Korea	2009	(Lee et al., 2010)
60	FJ705395	Germany	2004	(Greiser-Wilke et al., 2010)	253	GQ847594	Korea	2009	(Lee et al., 2010)
61	FJ705396	Germany	2004	(Greiser-Wilke et al., 2010)	254	GQ847595	Korea	2009	(Lee et al., 2010)
62	FJ705397	Germany	2004	(Greiser-Wilke et al., 2010)	255	GQ847596	Korea	2009	(Lee et al., 2010)
63	FJ705399	Germany	2004	(Greiser-Wilke et al., 2010)	256	GQ847597	Korea	2009	(Lee et al., 2010)
64	FJ705400	Germany	2004	(Greiser-Wilke et al., 2010)	257	JN315686	Korea	2009	(Han et al., 2011)
65	FJ705401	Germany	2004	(Greiser-Wilke et al., 2010)	258	JX570656	Korea	2010	This study
66	FJ705402	Germany	2004	(Greiser-Wilke et al., 2010)	259	JX570657	Korea	2010	This study
67	FJ705408	Germany	2004	(Greiser-Wilke et al., 2010)	260	JF681196	Korea	2010	(Kim et al., 2012)
68	FJ705410	Germany	2004	(Greiser-Wilke et al., 2010)	261	JF681194	Korea	2010	(Kim et al., 2012)
69	FJ705411	Germany	2004	(Greiser-Wilke et al., 2010)	262	JF681195	Korea	2010	(Kim et al., 2012)
70	FJ705418	Germany	2004	(Greiser-Wilke et al., 2010)	263	JF681197	Korea	2010	(Kim et al., 2012)
71	FJ705419	Germany	2004	(Greiser-Wilke et al., 2010)	264	JN696091	Korea	2010	(Kim et al., 2011b)
72	FJ705420	Germany	2004	(Greiser-Wilke et al., 2010)	265	JN696092	Korea	2010	(Kim et al., 2011b)
73	FJ705421	Germany	2004	(Greiser-Wilke et al., 2010)	266	JN696093	Korea	2010	(Kim et al., 2011b)
74	FJ705423	Germany	2004	(Greiser-Wilke et al., 2010)	267	JN696094	Korea	2010	(Kim et al., 2011b)
75	FJ705429	Germany	2004	(Greiser-Wilke et al., 2010)	268	JN696095	Korea	2010	(Kim et al., 2011b)
76	FJ705430	Germany	2004	(Greiser-Wilke et al., 2010)	269	JN696096	Korea	2010	(Kim et al., 2011b)
77	FJ705412	Germany	2005	(Greiser-Wilke et al., 2010)	270	JN696097	Korea	2010	(Kim et al., 2011b)
78	FJ705413	Germany	2005	(Greiser-Wilke et al., 2010)	271	JN696098	Korea	2010	(Kim et al., 2011b)
79	FJ705427	Germany	2005	(Greiser-Wilke et al., 2010)	272	JN696099	Korea	2010	(Kim et al., 2011b)
80	FJ705378	Germany	2006	(Greiser-Wilke et al., 2010)	273	JN696100	Korea	2010	(Kim et al., 2011b)
81	FJ705392	Germany	2006	(Greiser-Wilke et al., 2010)	274	JN696101	Korea	2010	(Kim et al., 2011b)
82	FJ705393	Germany	2006	(Greiser-Wilke et al., 2010)	275	JN696102	Korea	2010	(Kim et al., 2011b)
83	FJ705403	Germany	2006	(Greiser-Wilke et al., 2010)	276	JN696103	Korea	2010	(Kim et al., 2011b)
84	FJ705406	Germany	2006	(Greiser-Wilke et al., 2010)	277	JN696104	Korea	2010	(Kim et al., 2011b)
85	FJ705409	Germany	2006	(Greiser-Wilke et al., 2010)	278	JN696105	Korea	2010	(Kim et al., 2011b)
86	FJ705414	Germany	2006	(Greiser-Wilke et al., 2010)	279	JN696106	Korea	2010	(Kim et al., 2011b)
87	FJ705415	Germany	2006	(Greiser-Wilke et al., 2010)	280	JN696107	Korea	2010	(Kim et al., 2011b)
88	FJ705416	Germany	2006	(Greiser-Wilke et al., 2010)	281	JN696108	Korea	2010	(Kim et al., 2011b)
89	FJ705417	Germany	2006	(Greiser-Wilke et al., 2010)	282	JN696109	Korea	2010	(Kim et al., 2011b)

No.	*Sequence ID*	*Country*	*Year of isolation*	*Reference*	*No.*	*Sequence ID*	*Country*	*Year of isolation*	*Reference*
90	FJ705424	Germany	2006	(Greiser-Wilke et al., 2010)	283	JX570658	Korea	2010	This study
91	FJ705425	Germany	2006	(Greiser-Wilke et al., 2010)	284	JX570659	Korea	2010	This study
92	FJ705426	Germany	2006	(Greiser-Wilke et al., 2010)	285	JX570660	Korea	2011	This study
93	FJ705428	Germany	2006	(Greiser-Wilke et al., 2010)	286	JX570661	Korea	2011	This study
94	FJ705431	Germany	2006	(Greiser-Wilke et al., 2010)	287	JX570662	Korea	2011	This study
95	DQ366639	Hungary	2003	(Balka et al., 2008)	288	JX570663	Korea	2011	This study
96	DQ366640	Hungary	2003	(Balka et al., 2008)	289	JX570664	Korea	2011	This study
97	DQ366642	Hungary	2003	(Balka et al., 2008)	290	JX570665	Korea	2012	This study
98	DQ384979	Hungary	2003	(Balka et al., 2008)	291	JX570666	Korea	2012	This study
99	DQ384980	Hungary	2003	(Balka et al., 2008)	292	M96262	Netherlands	1991	(Meulenberg et al., 1993)
100	DQ384981	Hungary	2003	(Balka et al., 2008)	293	JF276435	Portugal	2006	(Darwich et al., 2011)
101	DQ384982	Hungary	2003	(Balka et al., 2008)	294	EU071240	Russia	1997	(Stadejek et al., 2008)
102	EF406337	Hungary	2003	(Balka et al., 2008)	295	EU071248	Russia	1998	(Stadejek et al., 2008)
103	EF406338	Hungary	2003	(Balka et al., 2008)	296	EU071239	Russia	2004	(Stadejek et al., 2008)
104	EF406339	Hungary	2003	(Balka et al., 2008)	297	EU071245	Russia	2004	(Stadejek et al., 2008)
105	EF406340	Hungary	2003	(Balka et al., 2008)	298	EU071230	Russia	2005	(Stadejek et al., 2008)
106	DQ366641	Hungary	2004	(Balka et al., 2008)	299	EU071233	Russia	2005	(Stadejek et al., 2008)
107	DQ366643	Hungary	2004	(Balka et al., 2008)	300	EU071235	Russia	2005	(Stadejek et al., 2008)
108	DQ366644	Hungary	2004	(Balka et al., 2008)	301	EU071237	Russia	2005	(Stadejek et al., 2008)
109	DQ366651	Hungary	2004	(Balka et al., 2008)	302	EU071238	Russia	2005	(Stadejek et al., 2008)
110	DQ366652	Hungary	2004	(Balka et al., 2008)	303	EU071244	Russia	2005	(Stadejek et al., 2008)
111	DQ366653	Hungary	2004	(Balka et al., 2008)	304	EU071250	Russia	2005	(Stadejek et al., 2008)
112	DQ366654	Hungary	2004	(Balka et al., 2008)	305	EU071251	Russia	2005	(Stadejek et al., 2008)
113	DQ366656	Hungary	2004	(Balka et al., 2008)	306	EU071231	Russia	2006	(Stadejek et al., 2008)
114	DQ366657	Hungary	2004	(Balka et al., 2008)	307	EU071232	Russia	2006	(Stadejek et al., 2008)
115	EF406341	Hungary	2004	(Balka et al., 2008)	308	EU071236	Russia	2006	(Stadejek et al., 2008)
116	EF406342	Hungary	2004	(Balka et al., 2008)	309	EU071241	Russia	2006	(Stadejek et al., 2008)
117	EF406343	Hungary	2004	(Balka et al., 2008)	310	EU071242	Russia	2006	(Stadejek et al., 2008)
118	EF406344	Hungary	2004	(Balka et al., 2008)	311	EU071243	Russia	2006	(Stadejek et al., 2008)
119	EF406345	Hungary	2004	(Balka et al., 2008)	312	EU071246	Russia	2006	(Stadejek et al., 2008)
120	EF406346	Hungary	2004	(Balka et al., 2008)	313	EU071247	Russia	2006	(Stadejek et al., 2008)
121	EF406347	Hungary	2004	(Balka et al., 2008)	314	EU071249	Russia	2006	(Stadejek et al., 2008)

No.	*Sequence ID*	*Country*	*Year of isolation*	*Reference*	*No.*	*Sequence ID*	*Country*	*Year of isolation*	*Reference*
122	DQ366645	Hungary	2005	(Balka et al., 2008)	315	AY035935	Spain	1991	(Forsberg et al., 2002)
123	DQ366646	Hungary	2005	(Balka et al., 2008)	316	AY035936	Spain	1991	(Forsberg et al., 2002)
124	DQ366647	Hungary	2005	(Balka et al., 2008)	317	JF276431	Spain	1992	(Darwich et al., 2011)
125	DQ366648	Hungary	2005	(Balka et al., 2008)	318	JF276430	Spain	2005	(Darwich et al., 2011)
126	DQ366649	Hungary	2005	(Balka et al., 2008)	319	JF276432	Spain	2005	(Darwich et al., 2011)
127	DQ366655	Hungary	2005	(Balka et al., 2008)	320	JF276433	Spain	2005	(Darwich et al., 2011)
128	DQ366658	Hungary	2005	(Balka et al., 2008)	321	AY297119	Thailand	2001	(Thanawongnuwech et al., 2004)
129	DQ384984	Hungary	2005	(Balka et al., 2008)	322	AY297120	Thailand	2001	(Thanawongnuwech et al., 2004)
130	DQ384985	Hungary	2005	(Balka et al., 2008)	323	DQ864705	Thailand	2001	(Amonsin et al., 2009)
131	DQ384986	Hungary	2005	(Balka et al., 2008)	324	AY297121	Thailand	2002	(Thanawongnuwech et al., 2004)
132	DQ384987	Hungary	2005	(Balka et al., 2008)	325	AY297122	Thailand	2002	(Thanawongnuwech et al., 2004)
133	DQ384988	Hungary	2005	(Balka et al., 2008)	326	AY297123	Thailand	2002	(Thanawongnuwech et al., 2004)
134	DQ384989	Hungary	2005	(Balka et al., 2008)	327	FJ908074	Thailand	2002	(Amonsin et al., 2009)
135	EF406348	Hungary	2005	(Balka et al., 2008)	328	AY297124	Thailand	2003	(Thanawongnuwech et al., 2004)
136	EF406349	Hungary	2005	(Balka et al., 2008)	329	FJ908075	Thailand	2008	(Amonsin et al., 2009)
137	EF406350	Hungary	2005	(Balka et al., 2008)	330	FJ908076	Thailand	2008	(Amonsin et al., 2009)
138	EF406351	Hungary	2005	(Balka et al., 2008)	331	JN002327	Thailand	2010	Unpublished
139	EF406352	Hungary	2005	(Balka et al., 2008)	332	JN002328	Thailand	2010	Unpublished
140	AY035926	Italy	1993	(Forsberg et al., 2002)	333	JN002329	Thailand	2010	Unpublished
141	AY035927	Italy	1993	(Forsberg et al., 2002)	334	JN002330	Thailand	2010	Unpublished
142	AY035929	Italy	1993	(Forsberg et al., 2002)	335	JN002331	Thailand	2010	Unpublished
143	AY035942	Italy	1993	(Forsberg et al., 2002)	336	JN002332	Thailand	2010	Unpublished
144	AY035932	Italy	1996	(Forsberg et al., 2002)	337	JN002333	Thailand	2010	Unpublished
145	AY035933	Italy	1996	(Forsberg et al., 2002)	338	JN002334	Thailand	2010	Unpublished
146	AY035943	Italy	1996	(Forsberg et al., 2002)	339	JN002335	Thailand	2010	Unpublished
147	AY035930	Italy	1997	(Forsberg et al., 2002)	340	JN002336	Thailand	2010	Unpublished
148	AY035931	Italy	1997	(Forsberg et al., 2002)	341	JN002337	Thailand	2010	Unpublished
149	AY035941	Italy	1997	(Forsberg et al., 2002)	342	JN002338	Thailand	2010	Unpublished
150	AY035934	Italy	1998	(Forsberg et al., 2002)	343	JN002339	Thailand	2010	Unpublished
151	AY739975	Italy	2002	(Pesente et al., 2006)	344	JN002340	Thailand	2010	Unpublished
152	AY739982	Italy	2002	(Pesente et al., 2006)	345	JN002341	Thailand	2010	Unpublished
153	AY739983	Italy	2002	(Pesente et al., 2006)	346	JN002342	Thailand	2010	Unpublished

No.	*Sequence ID*	*Country*	*Year of isolation*	*Reference*	*No.*	*Sequence ID*	*Country*	*Year of isolation*	*Reference*
154	AY740001	Italy	2002	(Pesente et al., 2006)	347	JN002344	Thailand	2010	Unpublished
155	AY740007	Italy	2002	(Pesente et al., 2006)	348	JN002345	Thailand	2010	Unpublished
156	AY740008	Italy	2002	(Pesente et al., 2006)	349	JN002346	Thailand	2010	Unpublished
157	AY730551	Italy	2003	(Pesente et al., 2006)	350	JN002347	Thailand	2010	Unpublished
158	AY739958	Italy	2003	(Pesente et al., 2006)	351	JN002348	Thailand	2010	Unpublished
159	AY739961	Italy	2003	(Pesente et al., 2006)	352	JN002350	Thailand	2010	Unpublished
160	AY739965	Italy	2003	(Pesente et al., 2006)	353	JN002351	Thailand	2010	Unpublished
161	AY739966	Italy	2003	(Pesente et al., 2006)	354	JN002352	Thailand	2010	Unpublished
162	AY739967	Italy	2003	(Pesente et al., 2006)	355	JN002353	Thailand	2010	Unpublished
163	AY739968	Italy	2003	(Pesente et al., 2006)	356	JN002354	Thailand	2010	Unpublished
164	AY739969	Italy	2003	(Pesente et al., 2006)	357	JN002355	Thailand	2010	Unpublished
165	AY739970	Italy	2003	(Pesente et al., 2006)	358	JN002356	Thailand	2010	Unpublished
166	AY739972	Italy	2003	(Pesente et al., 2006)	359	JN002357	Thailand	2010	Unpublished
167	AY739976	Italy	2003	(Pesente et al., 2006)	360	JN002358	Thailand	2010	Unpublished
168	AY739977	Italy	2003	(Pesente et al., 2006)	361	JN002359	Thailand	2010	Unpublished
169	AY739978	Italy	2003	(Pesente et al., 2006)	362	JN002360	Thailand	2010	Unpublished
170	AY739979	Italy	2003	(Pesente et al., 2006)	363	JN002362	Thailand	2010	Unpublished
171	AY739980	Italy	2003	(Pesente et al., 2006)	364	JN002363	Thailand	2010	Unpublished
172	AY739981	Italy	2003	(Pesente et al., 2006)	365	AY035938	UK	1991	(Forsberg et al., 2002)
173	AY739985	Italy	2003	(Pesente et al., 2006)	366	AY035939	UK	1992	(Forsberg et al., 2002)
174	AY739986	Italy	2003	(Pesente et al., 2006)	367	AY035940	UK	1992	(Forsberg et al., 2002)
175	AY739987	Italy	2003	(Pesente et al., 2006)	368	AY366525	USA	1999	(Ropp et al., 2004)
176	AY739988	Italy	2003	(Pesente et al., 2006)	369	AY422802	USA	2000	(Ropp et al., 2004)
177	AY739991	Italy	2003	(Pesente et al., 2006)	370	AY422803	USA	2000	(Ropp et al., 2004)
178	AY739992	Italy	2003	(Pesente et al., 2006)	371	AY422804	USA	2000	(Ropp et al., 2004)
179	AY739993	Italy	2003	(Pesente et al., 2006)	372	AY422805	USA	2000	(Ropp et al., 2004)
180	AY739995	Italy	2003	(Pesente et al., 2006)	373	AY422806	USA	2000	(Ropp et al., 2004)
181	AY739996	Italy	2003	(Pesente et al., 2006)	374	AY422807	USA	2000	(Ropp et al., 2004)
182	AY739997	Italy	2003	(Pesente et al., 2006)	375	AY422808	USA	2000	(Ropp et al., 2004)
183	AY739998	Italy	2003	(Pesente et al., 2006)	376	AY422809	USA	2000	(Ropp et al., 2004)
184	AY739999	Italy	2003	(Pesente et al., 2006)	377	AY395079	USA	2001	(Ropp et al., 2004)
185	AY740000	Italy	2003	(Pesente et al., 2006)	378	AY395080	USA	2001	(Ropp et al., 2004)

No.	*Sequence ID*	*Country*	*Year of isolation*	*Reference*
186	AY740004	Italy	2003	(Pesente et al., 2006)
187	AY740005	Italy	2003	(Pesente et al., 2006)
188	AY740006	Italy	2003	(Pesente et al., 2006)
189	AY740009	Italy	2003	(Pesente et al., 2006)
190	AY740011	Italy	2003	(Pesente et al., 2006)
191	AY740012	Italy	2003	(Pesente et al., 2006)
192	AY743932	Italy	2003	(Pesente et al., 2006)
193	AY743936	Italy	2003	(Pesente et al., 2006)

No.	*Sequence ID*	*Country*	*Year of isolation*	*Reference*
379	AY422799	USA	2001	(Ropp et al., 2004)
380	AY395078	USA	2002	(Ropp et al., 2004)
381	AY395081	USA	2002	(Ropp et al., 2004)
382	AY422798	USA	2002	(Ropp et al., 2004)
383	AY395074	USA	2003	(Ropp et al., 2004)
384	AY395075	USA	2003	(Ropp et al., 2004)
385	AY395076	USA	2003	(Ropp et al., 2004)

Supplementary Table S2.1. List of sequences used in analyses in chapter II. Sequences generated in this study are presented in bold. Sequences of Korean type 1 PRRSV formatted in red were used for analyses of evolutionary and epidemiological dynamics.

No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation
1	AY615790	Australia	NA	283	AY739962	Italy	2004	565	DQ324674	Poland	2005	847	JN862390	UK	2004
2	AY615791	Australia	NA	284	AY739989	Italy	2004	566	DQ324673	Poland	2005	848	JN862418	UK	2005
3	AY615788	Australia	NA	285	AY743935	Italy	2004	567	AY641473	Poland	NA	849	JN862401	UK	2005
4	AY615789	Australia	NA	286	AY743933	Italy	2004	568	JF730994	Poland	NA	850	JN862421	UK	2005
5	AY615793	Australia	NA	287	AY739959	Italy	2004	569	JF276435	Portugal	2006	851	JN862400	UK	2005
6	AY615792	Australia	NA	288	AY739963	Italy	2004	570	JX075094	Romania	2010	852	JN862436	UK	2005
7	AY615787	Australia	NA	289	AY740002	Italy	2004	571	JX099574	Romania	2011	853	JN862430	UK	2005
8	AY615786	Australia	NA	290	AY743934	Italy	2004	572	JX105430	Romania	2011	854	JN862422	UK	2005
9	AY875855	Austria	NA	291	AF486472	Italy	NA	573	JX099577	Romania	2011	855	JN862424	UK	2005
10	AY875861	Austria	NA	292	AF486468	Italy	NA	574	JX075096	Romania	2011	856	JN862427	UK	2005
11	AY875859	Austria	NA	293	AF486457	Italy	NA	575	JX090164	Romania	2011	857	JN862417	UK	2005
12	AY875853	Austria	NA	294	JF730997	Italy	NA	576	JX090165	Romania	2011	858	JN862416	UK	2005
13	AY875862	Austria	NA	295	JF730999	Italy	NA	577	JX090163	Romania	2011	859	JN862407	UK	2005
14	AY875860	Austria	NA	296	AF486482	Italy	NA	578	JX090166	Romania	2011	860	JN862405	UK	2005
15	AY875857	Austria	NA	297	AF486483	Italy	NA	579	JX090167	Romania	2011	861	JN862404	UK	2005
16	AY875858	Austria	NA	298	AF486484	Italy	NA	580	JX075097	Romania	2011	862	JN862411	UK	2005
17	AY875856	Austria	NA	299	AF486481	Italy	NA	581	JX099578	Romania	2011	863	JN862428	UK	2005

No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation
18	eu071229	Belarus	2002	300	AF486480	Italy	NA	582	JX099573	Romania	2011	864	JN862413	UK	2005
19	DQ324693	Belarus	2004	301	JF730998	Italy	NA	583	JX105431	Romania	2011	865	JN862399	UK	2005
20	DQ324692	Belarus	2004	302	AF486479	Italy	NA	584	JX099576	Romania	2011	866	JN862412	UK	2005
21	DQ324694	Belarus	2004	303	AF486458	Italy	NA	585	JX099572	Romania	2011	867	JN862410	UK	2005
22	DQ324695	Belarus	2004	304	AF486469	Italy	NA	586	JX099575	Romania	2011	868	JN862409	UK	2005
23	DQ324670	Belarus	2004	305	AF486471	Italy	NA	587	JX099579	Romania	2011	869	JN862402	UK	2005
24	DQ324669	Belarus	2004	306	AF486470	Italy	NA	588	EU071240	Russia	1997	870	JN862426	UK	2005
25	DQ324690	Belarus	2004	307	JF730996	Italy	NA	589	EU071248	Russia	1998	871	JN862398	UK	2005
26	DQ324691	Belarus	2004	308	AF486463	Italy	NA	590	EU071245	Russia	2004	872	JN862435	UK	2005
27	DQ324696	Belarus	2004	309	AF486459	Italy	NA	591	EU071239	Russia	2004	873	JN862434	UK	2005
28	DQ324697	Belarus	2004	310	AF486456	Italy	NA	592	EU071230	Russia	2005	874	JN862433	UK	2005
29	DQ324687	Belarus	2004	311	AF486454	Italy	NA	593	EU071238	Russia	2005	875	JN862406	UK	2005
30	DQ324686	Belarus	2004	312	AF486452	Italy	NA	594	EU071244	Russia	2005	876	JN862432	UK	2005
31	DQ324677	Belarus	2004	313	AF486474	Italy	NA	595	EU071251	Russia	2005	877	JN862425	UK	2005
32	DQ324683	Belarus	2004	314	AF486453	Italy	NA	596	EU071233	Russia	2005	878	JN862423	UK	2005
33	DQ324671	Belarus	2004	315	AF486460	Italy	NA	597	EU071234	Russia	2005	879	JN862431	UK	2005
34	DQ324672	Belarus	2004	316	AF486461	Italy	NA	598	EU071250	Russia	2005	880	JN862429	UK	2005
35	DQ324676	Belarus	2005	317	AF486467	Italy	NA	599	EU071235	Russia	2005	881	JN862408	UK	2005
36	DQ324689	Belarus	2005	318	AF486473	Italy	NA	600	EU071237	Russia	2005	882	JN862420	UK	2005
37	EU071228	Belarus	2006	319	AF486475	Italy	NA	601	EU071249	Russia	2006	883	JN862419	UK	2005
38	EU071227	Belarus	2006	320	AF486455	Italy	NA	602	EU071247	Russia	2006	884	JN862414	UK	2005

No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation
39	JF802085	Belarus	2007	321	AF486476	Italy	NA	603	EU071243	Russia	2006	885	JN862415	UK	2005
40	JN651734	Belarus	2009	322	AF486478	Italy	NA	604	EU071236	Russia	2006	886	JN862403	UK	2005
41	JN651735	Belarus	2010	323	AF486477	Italy	NA	605	EU071242	Russia	2006	887	JN862466	UK	2006
42	JN651736	Belarus	2010	324	AF486466	Italy	NA	606	EU071232	Russia	2006	888	JN862461	UK	2006
43	AY035901	Belgium	1992	325	AF486465	Italy	NA	607	EU071241	Russia	2006	889	JN862460	UK	2006
44	AY035900	Belgium	1992	326	AF486462	Italy	NA	608	EU071246	Russia	2006	890	JN862469	UK	2006
45	JF304781	Belgium	1994	327	AF486464	Italy	NA	609	EU071231	Russia	2006	891	JN862465	UK	2006
46	JF730990	Belgium	NA	328	EF031037	Korea	2005	610	AY035935	Spain	1991	892	JN862467	UK	2006
47	JF730991	Belgium	NA	329	DQ355821	Korea	2006	611	AY035936	Spain	1991	893	JN862443	UK	2006
48	HM755893	Belgium	NA	330	EF031038	Korea	2006	612	JF276431	Spain	1992	894	JN862440	UK	2006
49	HM755894	Belgium	NA	331	EF031040	Korea	2006	613	DQ345737	Spain	1993	895	JN862459	UK	2006
50	HM755897	Belgium	NA	332	EF031039	Korea	2006	614	DQ345738	Spain	1994	896	JN862453	UK	2006
51	HM755889	Belgium	NA	333	EF031042	Korea	2006	615	JF276432	Spain	2005	897	JN862454	UK	2006
52	HM755891	Belgium	NA	334	EF031041	Korea	2006	616	JF276430	Spain	2005	898	JN862444	UK	2006
53	HM755890	Belgium	NA	335	EF031043	Korea	2006	617	JF276433	Spain	2005	899	JN862456	UK	2006
54	HM755892	Belgium	NA	336	GQ847573	Korea	2006	618	AF495517	Spain	NA	900	JN862448	UK	2006
55	HM755896	Belgium	NA	337	EF031044	Korea	2006	619	AF495518	Spain	NA	901	JN862447	UK	2006
56	HM755895	Belgium	NA	338	GU325643	Korea	2007	620	DQ009636	Spain	NA	902	JN862451	UK	2006
57	JF730992	Belgium	NA	339	GU325642	Korea	2007	621	DQ009638	Spain	NA	903	JN862450	UK	2006
58	GU047344	China	2006	340	FJ349261	Korea	2007	622	DQ345743	Spain	NA	904	JN862439	UK	2006
59	GU047342	China	2009	341	GU325645	Korea	2008	623	JF730969	Spain	NA	905	JN862449	UK	2006

No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation
60	GU047341	China	2009	342	GU325644	Korea	2008	624	JF730975	Spain	NA	906	JN862455	UK	2006
61	GU047340	China	2009	343	GU325646	Korea	2008	625	DQ345755	Spain	NA	907	JN862438	UK	2006
62	GU047343	China	2009	344	GQ847574	Korea	2008	626	AF495512	Spain	NA	908	JN862442	UK	2006
63	AY633973	China	NA	345	GQ847579	Korea	2008	627	AF495520	Spain	NA	909	JN862437	UK	2006
64	AF253537	Czech	1995	346	GQ847580	Korea	2008	628	AF495519	Spain	NA	910	JN862441	UK	2006
65	AF253531	Czech	1996	347	GQ847586	Korea	2008	629	DQ345753	Spain	NA	911	JN862457	UK	2006
66	AF253532	Czech	1996	348	GQ847587	Korea	2008	630	JF730973	Spain	NA	912	JN862452	UK	2006
67	AF253534	Czech	1998	349	GQ847581	Korea	2008	631	AF495502	Spain	NA	913	JN862445	UK	2006
68	AF253535	Czech	1998	350	GQ847590	Korea	2008	632	DQ009640	Spain	NA	914	JN862468	UK	2006
69	EU071226	Czech	NA	351	GQ847583	Korea	2008	633	DQ009639	Spain	NA	915	JN862464	UK	2006
70	JF730993	Czech	NA	352	GQ847582	Korea	2008	634	EF429102	Spain	NA	916	JN862462	UK	2006
71	AF253536	Czech	NA	353	GQ847592	Korea	2008	635	DQ345745	Spain	NA	917	JN862463	UK	2006
72	AF253533	Czech	NA	354	GQ847589	Korea	2008	636	EF429101	Spain	NA	918	JN862458	UK	2006
73	AY035916	Denmark	1992	355	GQ847591	Korea	2008	637	DQ345733	Spain	NA	919	JN862446	UK	2006
74	AY035913	Denmark	1992	356	GU325648	Korea	2008	638	JF730962	Spain	NA	920	JN862486	UK	2007
75	AY035944	Denmark	1992	357	FJ972763	Korea	2008	639	DQ345730	Spain	NA	921	JN862484	UK	2007
76	AY035906	Denmark	1993	358	FJ972764	Korea	2008	640	JF730964	Spain	NA	922	JN862485	UK	2007
77	AY035909	Denmark	1993	359	GQ847588	Korea	2008	641	JF730971	Spain	NA	923	JN862483	UK	2007
78	AY035903	Denmark	1995	360	GQ847578	Korea	2008	642	DQ345749	Spain	NA	924	JN862479	UK	2007
79	AY035902	Denmark	1995	361	FJ972762	Korea	2008	643	EF429110	Spain	NA	925	JN862489	UK	2007
80	AY035917	Denmark	1995	362	GQ847575	Korea	2008	644	EF429112	Spain	NA	926	JN862478	UK	2007

No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation
81	AY035905	Denmark	1996	363	FJ972766	Korea	2008	645	AF495516	Spain	NA	927	JN862490	UK	2007
82	AY035904	Denmark	1996	364	FJ972765	Korea	2008	646	AF495515	Spain	NA	928	JN862488	UK	2007
83	AY035908	Denmark	1997	365	GQ847584	Korea	2008	647	DQ345732	Spain	NA	929	JN862481	UK	2007
84	AY035910	Denmark	1997	366	GQ847585	Korea	2008	648	JF730963	Spain	NA	930	JN862482	UK	2007
85	AY035907	Denmark	1997	367	GU325647	Korea	2008	649	DQ345728	Spain	NA	931	JN862473	UK	2007
86	AY035911	Denmark	1998	368	GQ847576	Korea	2008	650	JF730984	Spain	NA	932	JN862472	UK	2007
87	AY035912	Denmark	1998	369	GQ847577	Korea	2008	651	JF730961	Spain	NA	933	JN862474	UK	2007
88	AY035928	Denmark	NA	370	GQ847597	Korea	2009	652	DQ345729	Spain	NA	934	JN862475	UK	2007
89	AY035914	Denmark	NA	371	GQ847593	Korea	2009	653	EF429115	Spain	NA	935	JN862480	UK	2007
90	AY035915	Denmark	NA	372	GQ847594	Korea	2009	654	DQ064788	Spain	NA	936	JN862476	UK	2007
91	AF315709	Denmark	NA	373	GQ847595	Korea	2009	655	af378820	Spain	NA	937	JN862470	UK	2007
92	AF315708	Denmark	NA	374	GQ847596	Korea	2009	656	DQ324681	Spain	NA	938	JN862471	UK	2007
93	AF315707	Denmark	NA	375	JF681194	Korea	2010	657	AF495504	Spain	NA	939	JN862491	UK	2007
94	AF315703	Denmark	NA	376	JN696106	Korea	2010	658	DQ345726	Spain	NA	940	JN862487	UK	2007
95	AF315700	Denmark	NA	377	JN696104	Korea	2010	659	JF730979	Spain	NA	941	JN862477	UK	2007
96	AF315702	Denmark	NA	378	JN696097	Korea	2010	660	JF730983	Spain	NA	942	JN862493	UK	2008
97	AF315704	Denmark	NA	379	JF681195	Korea	2010	661	EF429113	Spain	NA	943	JN862494	UK	2008
98	AF315705	Denmark	NA	380	JN696096	Korea	2010	662	JF730967	Spain	NA	944	JN862492	UK	2008
99	AF315701	Denmark	NA	381	JN696091	Korea	2010	663	DQ345740	Spain	NA	945	JN862503	UK	2008
100	AF315706	Denmark	NA	382	JN696094	Korea	2010	664	JF730972	Spain	NA	946	JN862507	UK	2008
101	AY035937	Denmark	NA	383	JN696093	Korea	2010	665	JF730980	Spain	NA	947	JN862505	UK	2008

No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation
102	AY035918	France	1991	384	JN696092	Korea	2010	666	DQ009642	Spain	NA	948	JN862506	UK	2008
103	AY035919	France	1992	385	JN696108	Korea	2010	667	EF429114	Spain	NA	949	JN862504	UK	2008
104	AY035920	France	1993	386	JN696101	Korea	2010	668	DQ345725	Spain	NA	950	JN862498	UK	2008
105	AY035924	Germany	1993	387	JN696100	Korea	2010	669	EF429118	Spain	NA	951	JN862499	UK	2008
106	AY035923	Germany	1993	388	JN696095	Korea	2010	670	DQ009647	Spain	NA	952	JN862508	UK	2008
107	AY035922	Germany	1993	389	JN696102	Korea	2010	671	AF495503	Spain	NA	953	JN862500	UK	2008
108	AY035921	Germany	1993	390	JN696098	Korea	2010	672	AF495510	Spain	NA	954	JN862502	UK	2008
109	AY035925	Germany	1993	391	JN696099	Korea	2010	673	DQ324668	Spain	NA	955	JN862509	UK	2008
110	JF276434	Germany	1996	392	JN696105	Korea	2010	674	DQ345747	Spain	NA	956	JN862501	UK	2008
111	GQ451675	Germany	1997	393	JN696107	Korea	2010	675	JF730970	Spain	NA	957	JN862497	UK	2008
112	FJ705375	Germany	2003	394	JN696103	Korea	2010	676	EF429117	Spain	NA	958	JN862495	UK	2008
113	FJ705373	Germany	2003	395	JN696109	Korea	2010	677	EF429103	Spain	NA	959	JN862496	UK	2008
114	FJ705374	Germany	2003	396	JX988619	Korea	2010	678	AF495505	Spain	NA	960	JN862510	UK	2009
115	FJ705376	Germany	2003	397	**S5744_3**	**Korea**	**2010**	679	AF495509	Spain	NA	961	AF378799	UK	NA
116	FJ705377	Germany	2003	398	JF681197	Korea	2010	680	AF495513	Spain	NA	962	AY366525	USA	1999
117	FJ705391	Germany	2004	399	JF681196	Korea	2010	681	AF495508	Spain	NA	963	AY422803	USA	2000
118	FJ705418	Germany	2004	400	KC288105	Korea	2010	682	EF429100	Spain	NA	964	AY422804	USA	2000
119	FJ705421	Germany	2004	401	**G10_01078_1**	**Korea**	**2010**	683	DQ345741	Spain	NA	965	AY422802	USA	2000
120	FJ705408	Germany	2004	402	**G10_01078_2**	**Korea**	**2010**	684	DQ345736	Spain	NA	966	AY422806	USA	2000
121	FJ705423	Germany	2004	403	JX570659	Korea	2010	685	DQ009634	Spain	NA	967	AY422807	USA	2000
122	FJ705381	Germany	2004	404	JX393303	Korea	2010	686	DQ345752	Spain	NA	968	AY422808	USA	2000

No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation
123	FJ705380	Germany	2004	405	JX988618	Korea	2010	687	EF429116	Spain	NA	969	AY422805	USA	2000
124	FJ705430	Germany	2004	406	JX570656	Korea	2010	688	EF429105	Spain	NA	970	AY422809	USA	2000
125	FJ705399	Germany	2004	407	JX570657	Korea	2010	689	EF429106	Spain	NA	971	AY395079	USA	2001
126	FJ705429	Germany	2004	408	JX988617	Korea	2010	690	EF429107	Spain	NA	972	AY395080	USA	2001
127	FJ705410	Germany	2004	409	JN411262	Korea	2010	691	DQ009646	Spain	NA	973	AY422799	USA	2001
128	FJ705401	Germany	2004	410	JX570658	Korea	2010	692	EF429099	Spain	NA	974	AY422798	USA	2002
129	FJ705411	Germany	2004	411	**GC13**	**Korea**	**2011**	693	AF495521	Spain	NA	975	AY395081	USA	2002
130	FJ705395	Germany	2004	412	**A4331**	**Korea**	**2011**	694	DQ009633	Spain	NA	976	AY395078	USA	2002
131	FJ705397	Germany	2004	413	JX570663	Korea	2011	695	DQ009627	Spain	NA	977	AY395075	USA	2003
132	FJ705394	Germany	2004	414	JX570664	Korea	2011	696	DQ009631	Spain	NA	978	AY395076	USA	2003
133	FJ705396	Germany	2004	415	**A5702**	**Korea**	**2011**	697	DQ009635	Spain	NA	979	AY395074	USA	2003
134	FJ705419	Germany	2004	416	**CG11**	**Korea**	**2011**	698	DQ009632	Spain	NA	980	DQ477805	USA	NA
135	FJ705420	Germany	2004	417	JX570661	Korea	2011	699	DQ009629	Spain	NA	981	DQ475447	USA	NA
136	FJ705400	Germany	2004	418	JX570660	Korea	2011	700	DQ009625	Spain	NA	982	EU759749	USA	NA
137	FJ705385	Germany	2004	419	JX570662	Korea	2011	701	DQ009630	Spain	NA	983	EU758457	USA	NA
138	FJ705386	Germany	2004	420	**CP12_106_13**	**Korea**	**2012**	702	DQ009628	Spain	NA	984	EU759429	USA	NA
139	FJ705383	Germany	2004	421	**CP12_106_15**	**Korea**	**2012**	703	DQ009643	Spain	NA	985	DQ475490	USA	NA
140	FJ705384	Germany	2004	422	**CP12_106_12**	**Korea**	**2012**	704	AF495514	Spain	NA	986	EU758565	USA	NA
141	FJ705402	Germany	2004	423	**CP12_106_1**	**Korea**	**2012**	705	DQ345734	Spain	NA	987	EU757447	USA	NA
142	FJ705412	Germany	2005	424	**CP12_106_8**	**Korea**	**2012**	706	JF730965	Spain	NA	988	EU757420	USA	NA
143	FJ705427	Germany	2005	425	**CP12_421_12**	**Korea**	**2012**	707	AF495507	Spain	NA	989	EU755686	USA	NA

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144	FJ705413	Germany	2005	426	CP12_421_15 1	Korea	2012	708	AF495506	Spain	NA	990	EU757317	USA	NA
145	FJ705393	Germany	2006	427	CP12_421_15_ 2	Korea	2012	709	DQ345744	Spain	NA	991	EU757401	USA	NA
146	FJ705392	Germany	2006	428	CP12_421_11	Korea	2012	710	DQ345735	Spain	NA	992	AY395077	USA	NA
147	FJ705431	Germany	2006	429	CP12_170_24	Korea	2012	711	DQ064787	Spain	NA	993	EU756552	USA	NA
148	FJ705424	Germany	2006	430	CP12_170_27	Korea	2012	712	DQ345727	Spain	NA	994	EU756550	USA	NA
149	FJ705428	Germany	2006	431	CP12_141_2	Korea	2012	713	DQ009637	Spain	NA	995	EU756393	USA	NA
150	FJ705409	Germany	2006	432	CP12_68_7	Korea	2012	714	DQ009641	Spain	NA	996	EU759494	USA	NA
151	FJ705426	Germany	2006	433	CP12_106_14	Korea	2012	715	EF429108	Spain	NA	997	EU758370	USA	NA
152	FJ705417	Germany	2006	434	CP12_25_13	Korea	2012	716	DQ345748	Spain	NA	998	EU759117	USA	NA
153	FJ705414	Germany	2006	435	CP12_357_11	Korea	2012	717	JF730982	Spain	NA	999	EU759098	USA	NA
154	FJ705416	Germany	2006	436	CP12_210_22	Korea	2012	718	DQ345750	Spain	NA	1000	EU759604	USA	NA
155	FJ705415	Germany	2006	437	CP12_210_24	Korea	2012	719	DQ345742	Spain	NA	1001	EU759686	USA	NA
156	FJ705425	Germany	2006	438	CP12_523_13	Korea	2012	720	JF730968	Spain	NA	1002	EU759349	USA	NA
157	FJ705378	Germany	2006	439	CP12_314_25	Korea	2012	721	EF429111	Spain	NA	1003	EU759254	USA	NA
158	FJ705403	Germany	2006	440	CP12_357_8	Korea	2012	722	DQ345751	Spain	NA	1004	EU759275	USA	NA
159	FJ705406	Germany	2006	441	CP12_358_8	Korea	2012	723	JF730976	Spain	NA	1005	EU758966	USA	NA
160	JN651729	Germany	NA	442	CP12_141_6	Korea	2012	724	JF730977	Spain	NA	1006	EU759012	USA	NA
161	JN651731	Germany	NA	443	CP12_141_1	Korea	2012	725	JF730978	Spain	NA	1007	EU759277	USA	NA
162	JF730985	Germany	NA	444	CP12_210_27	Korea	2012	726	DQ009644	Spain	NA	1008	EU759256	USA	NA
163	JN651728	Germany	NA	445	S7691_4	Korea	2012	727	DQ345754	Spain	NA	1009	DQ476364	USA	NA
164	AF378798	Germany	NA	446	A7106	Korea	2012	728	JF730974	Spain	NA	1010	EU757746	USA	NA

No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation
165	JN651739	Germany	NA	447	S7676_3	Korea	2012	729	AF495511	Spain	NA	1011	EU757980	USA	NA
166	JN651730	Germany	NA	448	S7572_3	Korea	2012	730	DQ009626	Spain	NA	1012	EF175566	USA	NA
167	JF730987	Germany	NA	449	CP12_348_4	Korea	2012	731	DQ345739	Spain	NA	1013	EU758172	USA	NA
168	AF378797	Germany	NA	450	CP12_511_8	Korea	2012	732	JF730966	Spain	NA	1014	EU758411	USA	NA
169	JF730986	Germany	NA	451	CP12_511_6	Korea	2012	733	AF495499	Spain	NA	1015	EU758401	USA	NA
170	JN651740	Germany	NA	452	CP12_525_4	Korea	2012	734	AF495500	Spain	NA	1016	EU759202	USA	NA
171	JN651738	Germany	NA	453	CP12_504_4	Korea	2012	735	DQ345731	Spain	NA	1017	EU758754	USA	NA
172	JN651732	Germany	NA	454	A6848	Korea	2012	736	EF429109	Spain	NA	1018	EU758744	USA	NA
173	JN651737	Germany	NA	455	A6846	Korea	2012	737	AF495501	Spain	NA	1019	EU758850	USA	NA
174	DQ366642	Hungary	2003	456	S7678_3	Korea	2012	738	EF429104	Spain	NA	1020	EU758871	USA	NA
175	DQ366640	Hungary	2003	457	S7678_2	Korea	2012	739	JF730981	Spain	NA	1021	EU758851	USA	NA
176	EF406340	Hungary	2003	458	A6847_1	Korea	2012	740	DQ345746	Spain	NA	1022	EU758856	USA	NA
177	DQ366639	Hungary	2003	459	A6847_2	Korea	2012	741	AY297120	Thailand	2001	1023	DQ477659	USA	NA
178	EF406339	Hungary	2003	460	S7662_3	Korea	2012	742	AY297119	Thailand	2001	1024	EU755691	USA	NA
179	EF406337	Hungary	2003	461	PF2329_2	Korea	2012	743	DQ864705	Thailand	2001	1025	DQ476358	USA	NA
180	EF406338	Hungary	2003	462	A7103	Korea	2012	744	FJ908074	Thailand	2002	1026	DQ475238	USA	NA
181	DQ384982	Hungary	2003	463	A7107	Korea	2012	745	AY297123	Thailand	2002	1027	EU755430	USA	NA
182	DQ384980	Hungary	2003	464	A7108	Korea	2012	746	AY297121	Thailand	2002	1028	EU758692	USA	NA
183	DQ384979	Hungary	2003	465	A7104	Korea	2012	747	AY297122	Thailand	2002	1029	EU758691	USA	NA
184	DQ384981	Hungary	2003	466	JX570666	Korea	2012	748	AY297124	Thailand	2003	1030	EU755436	USA	NA
185	EF406347	Hungary	2004	467	M1494_9_1	Korea	2012	749	FJ908075	Thailand	2008	1031	EU758949	USA	NA

No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation
186	EF406341	Hungary	2004	468	M1494_9_2	Korea	2012	750	FJ908076	Thailand	2008	1032	EU758852	USA	NA
187	DQ366641	Hungary	2004	469	S7689_2	Korea	2012	751	JN002346	Thailand	2010	1033	EU759022	USA	NA
188	DQ366653	Hungary	2004	470	S7379_5	Korea	2012	752	JN002348	Thailand	2010	1034	EU758531	USA	NA
189	EF406345	Hungary	2004	471	S7379_4	Korea	2012	753	JN002347	Thailand	2010	1035	EU758927	USA	NA
190	EF406343	Hungary	2004	472	S7657_7	Korea	2012	754	JN002358	Thailand	2010	1036	EU758488	USA	NA
191	DQ366643	Hungary	2004	473	S7412_1	Korea	2012	755	JN002359	Thailand	2010	1037	EU759084	USA	NA
192	EF406342	Hungary	2004	474	S7731_4	Korea	2012	756	JN002360	Thailand	2010	1038	EU759110	USA	NA
193	EF406344	Hungary	2004	475	S7358_2	Korea	2012	757	JN002351	Thailand	2010	1039	EU759153	USA	NA
194	DQ366644	Hungary	2004	476	CP12_484_1	Korea	2012	758	JN002357	Thailand	2010	1040	EU759148	USA	NA
195	EF406346	Hungary	2004	477	CP12_484_2	Korea	2012	759	JN002353	Thailand	2010	1041	EU759149	USA	NA
196	DQ366656	Hungary	2004	478	A7098	Korea	2012	760	JN002355	Thailand	2010	1042	EU759124	USA	NA
197	DQ366657	Hungary	2004	479	A7097	Korea	2012	761	JN002356	Thailand	2010	1043	EU759204	USA	NA
198	DQ366652	Hungary	2004	480	S7658_3	Korea	2012	762	JN002354	Thailand	2010	1044	EU758901	USA	NA
199	DQ366651	Hungary	2004	481	S7734_2	Korea	2012	763	JN002352	Thailand	2010	1045	EU759775	USA	NA
200	DQ366654	Hungary	2004	482	S7658_4	Korea	2012	764	JN002363	Thailand	2010	1046	EU759959	USA	NA
201	EF406352	Hungary	2005	483	A6991	Korea	2012	765	JN002362	Thailand	2010	1047	EU759776	USA	NA
202	DQ384989	Hungary	2005	484	A6989	Korea	2012	766	JN002350	Thailand	2010	1048	EU759897	USA	NA
203	EF406350	Hungary	2005	485	S7688_1	Korea	2012	767	JN002339	Thailand	2010	1049	EU755501	USA	NA
204	DQ366648	Hungary	2005	486	S7688_2	Korea	2012	768	JN002340	Thailand	2010	1050	EU757125	USA	NA
205	EF406348	Hungary	2005	487	S7657_5	Korea	2012	769	JN002341	Thailand	2010	1051	EU758485	USA	NA
206	DQ384987	Hungary	2005	488	S7683_3	Korea	2012	770	JN002332	Thailand	2010	1052	EF175568	USA	NA

No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation
207	EF406349	Hungary	2005	489	A6990	Korea	2012	771	JN002331	Thailand	2010	1053	EU758643	USA	NA
208	DQ384988	Hungary	2005	490	S7683_4	Korea	2012	772	JN002330	Thailand	2010	1054	EU758606	USA	NA
209	DQ366647	Hungary	2005	491	M1482	Korea	2012	773	JN002327	Thailand	2010	1055	DQ478091	USA	NA
210	DQ384986	Hungary	2005	492	CP12_304_4	Korea	2012	774	JN002337	Thailand	2010	1056	EU755767	USA	NA
211	DQ366649	Hungary	2005	493	CP12_427_11	Korea	2012	775	JN002329	Thailand	2010	1057	EU759889	USA	NA
212	DQ384985	Hungary	2005	494	CP12_431_11	Korea	2012	776	JN002336	Thailand	2010	1058	EU759890	USA	NA
213	EF406351	Hungary	2005	495	CP12_431_10	Korea	2012	777	JN002335	Thailand	2010	1059	EU759971	USA	NA
214	DQ366645	Hungary	2005	496	S7736_4	Korea	2012	778	JN002333	Thailand	2010	1060	EU755469	USA	NA
215	DQ366658	Hungary	2005	497	S7728_8	Korea	2012	779	JN002334	Thailand	2010	1061	EU759152	USA	NA
216	DQ366655	Hungary	2005	498	A7031	Korea	2012	780	JN002338	Thailand	2010	1062	EU759097	USA	NA
217	DQ366646	Hungary	2005	499	CP12_439_24	Korea	2012	781	JN002328	Thailand	2010	1063	EU759536	USA	NA
218	DQ384984	Hungary	2005	500	CP12_439_25	Korea	2012	782	JN002342	Thailand	2010	1064	EU758595	USA	NA
219	DQ384983	Hungary	NA	501	CP12_439_22	Korea	2012	783	JN002345	Thailand	2010	1065	EU758633	USA	NA
220	JF730995	Hungary	NA	502	CP12_439_17	Korea	2012	784	JN002344	Thailand	2010	1066	EU758561	USA	NA
221	AY035929	Italy	1993	503	CP12_439_6	Korea	2012	785	JN848616	Thailand	NA	1067	EU758608	USA	NA
222	AY035942	Italy	1993	504	CP12_439_30	Korea	2012	786	JN848609	Thailand	NA	1068	EU757917	USA	NA
223	AY035927	Italy	1993	505	CP12_439_26	Korea	2012	787	JN848601	Thailand	NA	1069	EU757955	USA	NA
224	AY035926	Italy	1993	506	CP12_439_29	Korea	2012	788	JN848605	Thailand	NA	1070	EU757936	USA	NA
225	AY035943	Italy	1996	507	CP12_439_27	Korea	2012	789	JN848624	Thailand	NA	1071	EU757843	USA	NA
226	AY035932	Italy	1996	508	CP12_439_28	Korea	2012	790	JN848627	Thailand	NA	1072	EU758352	USA	NA
227	AY035933	Italy	1996	509	JX570665	Korea	2012	791	JN002361	Thailand	NA	1073	EU759559	USA	NA

No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation
228	AY035930	Italy	1997	510	CP12_502_13	Korea	2012	792	JN848619	Thailand	NA	1074	EU757741	USA	NA
229	AY035931	Italy	1997	511	S7734_3	Korea	2012	793	JN848632	Thailand	NA	1075	EU758890	USA	NA
230	AY035941	Italy	1997	512	A7207	Korea	2013	794	JN848633	Thailand	NA	1076	EU759930	USA	NA
231	AY035934	Italy	1998	513	S7837_4	Korea	2013	795	JN848602	Thailand	NA	1077	EU758573	USA	NA
232	AY739983	Italy	2002	514	S7813_1	Korea	2013	796	JN848629	Thailand	NA	1078	EU758502	USA	NA
233	AY739982	Italy	2002	515	CP13_3_4	Korea	2013	797	JN848630	Thailand	NA	1079	EU758576	USA	NA
234	AY740007	Italy	2002	516	P47	Korea	2013	798	JN848623	Thailand	NA	1080	EU758813	USA	NA
235	AY740008	Italy	2002	517	P48	Korea	2013	799	JN848604	Thailand	NA	1081	EU758555	USA	NA
236	AY739975	Italy	2002	518	CP13_8_1	Korea	2013	800	JN848634	Thailand	NA	1082	EU759373	USA	NA
237	AY740001	Italy	2002	519	S7777_2	Korea	2013	801	JN848620	Thailand	NA	1083	EU758819	USA	NA
238	AY739985	Italy	2003	520	P78	Korea	2013	802	JN848611	Thailand	NA	1084	EU759107	USA	NA
239	AY740012	Italy	2003	521	S7836_2	Korea	2013	803	JN848607	Thailand	NA	1085	EU758921	USA	NA
240	AY739976	Italy	2003	522	S7799_5	Korea	2013	804	JN848628	Thailand	NA	1086	EU759001	USA	NA
241	AY739979	Italy	2003	523	M1510_9	Korea	2013	805	JN848631	Thailand	NA	1087	EU758836	USA	NA
242	AY743937	Italy	2003	524	CP13_18_9	Korea	2013	806	JN848618	Thailand	NA	1088	EU759955	USA	NA
243	AY739972	Italy	2003	525	CP13_50_2	Korea	2013	807	JN848613	Thailand	NA	1089	EU758554	USA	NA
244	AY739969	Italy	2003	526	CP13_50_1	Korea	2013	808	JN848625	Thailand	NA	1090	EU758574	USA	NA
245	AY739980	Italy	2003	527	S7779_3	Korea	2013	809	JN848608	Thailand	NA	1091	EU759554	USA	NA
246	AY739981	Italy	2003	528	S7779_2	Korea	2013	810	JN848612	Thailand	NA	1092	EU758992	USA	NA
247	AY739965	Italy	2003	529	S7843_3	Korea	2013	811	JN848617	Thailand	NA	1093	EU758977	USA	NA
248	AY739961	Italy	2003	530	P23	Korea	2013	812	JN002349	Thailand	NA	1094	EU758910	USA	NA

No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation
249	AY739997	Italy	2003	531	**S7817_5**	**Korea**	**2013**	813	JN848622	Thailand	NA	1095	EU759205	USA	NA
250	AY739978	Italy	2003	532	af378802	Lithuania	2000	814	JN848615	Thailand	NA	1096	EU758575	USA	NA
251	AY739966	Italy	2003	533	DQ324667	Lithuania	2000	815	JN848606	Thailand	NA	1097	EF175572	USA	NA
252	AY739998	Italy	2003	534	af378800	Lithuania	2000	816	JN848610	Thailand	NA	1098	EF175570	USA	NA
253	AY743936	Italy	2003	535	af378801	Lithuania	2000	817	JN848621	Thailand	NA	1099	EU757660	USA	NA
254	AY740011	Italy	2003	536	af378803	Lithuania	2000	818	JN848603	Thailand	NA	1100	DQ476800	USA	NA
255	AY739999	Italy	2003	537	DQ324682	Lithuania	2000	819	JN848626	Thailand	NA	1101	DQ476779	USA	NA
256	AY739988	Italy	2003	538	JN651733	Lithuania	2002	820	JN848614	Thailand	NA	1102	DQ478339	USA	NA
257	AY739977	Italy	2003	539	M96262	Netherlands	1991	821	JN002343	Thailand	NA	1103	EU759617	USA	NA
258	AY739992	Italy	2003	540	JF730988	Netherlands	NA	822	JN862379	UK	1991	1104	EU759758	USA	NA
259	AY739991	Italy	2003	541	af378819	Netherlands	NA	823	JN862380	UK	1991	1105	EU758389	USA	NA
260	AY739968	Italy	2003	542	DQ324678	Netherlands	NA	824	JN862377	UK	1991	1106	EU757960	USA	NA
261	AY730551	Italy	2003	543	JF730989	Netherlands	NA	825	JN862375	UK	1991	1107	DQ475472	USA	NA
262	AY739958	Italy	2003	544	af378805	Poland	1994	826	AY035938	UK	1991	1108	EU758262	USA	NA
263	AY739995	Italy	2003	545	af378804	Poland	1994	827	JN862376	UK	1991	1109	EU758271	USA	NA
264	AY739967	Italy	2003	546	af378807	Poland	1996	828	JN862378	UK	1991	1110	EU758248	USA	NA
265	AY739970	Italy	2003	547	af378806	Poland	1996	829	JN862383	UK	1992	1111	EU758247	USA	NA
266	AY739996	Italy	2003	548	af378811	Poland	1997	830	JN862381	UK	1992	1112	EU758261	USA	NA
267	AY749417	Italy	2003	549	af378812	Poland	1997	831	AY035940	UK	1992	1113	EU758589	USA	NA
268	AY739986	Italy	2003	550	af378808	Poland	1997	832	AY035939	UK	1992	1114	EU759015	USA	NA
269	AY739993	Italy	2003	551	af378810	Poland	1997	833	JN862384	UK	1992	1115	EU759351	USA	NA
270	AY739987	Italy	2003	552	af378809	Poland	1997	834	JN862382	UK	1992	1116	EU759350	USA	NA

No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation	No.	Isolate	Country	Year of isolation
271	AY740006	Italy	2003	553	af378813	Poland	1997	835	JN862385	UK	1993	1117	EU758817	USA	NA
272	AY740005	Italy	2003	554	af378814	Poland	1998	836	JN862386	UK	1994	1118	EU758954	USA	NA
273	AY740004	Italy	2003	555	af378817	Poland	2000	837	JN862387	UK	1994	1119	EU758891	USA	NA
274	AY740009	Italy	2003	556	af378816	Poland	2000	838	JN862388	UK	2003	1120	EU759048	USA	NA
275	AY740000	Italy	2003	557	af378815	Poland	2000	839	JN862396	UK	2004	1121	EU758859	USA	NA
276	AY743932	Italy	2003	558	af378818	Poland	2001	840	JN862397	UK	2004	1122	EU758562	USA	NA
277	AY739984	Italy	2004	559	DQ324685	Poland	2004	841	JN862392	UK	2004	1123	EU758084	USA	NA
278	AY739964	Italy	2004	560	DQ324684	Poland	2004	842	JN862391	UK	2004	1124	EU755488	USA	NA
279	AY739960	Italy	2004	561	DQ324675	Poland	2005	843	JN862394	UK	2004	1125	AF514803	USA	NA
280	AY739973	Italy	2004	562	DQ324688	Poland	2005	844	JN862389	UK	2004	1126	EU758974	USA	NA
281	AY739971	Italy	2004	563	DQ324680	Poland	2005	845	JN862395	UK	2004	1127	L04493_Boxmeer10	NA	NA
282	AY739990	Italy	2004	564	DQ324679	Poland	2005	846	JN862393	UK	2004				

NA: not availble

Supplementary Table S3.1. List of sequences of the typical and highly pathogenic clades of type 2 PRRSV used in chapter III

No.	GenBank[a]	Country of origin	Year of isolation	Classification		No.	GenBank[a]	Country of origin	Year of isolation	Classification	
				Lineage[b]	*Clade*[c]					*Lineage*[b]	*Clade*[c]
1	DQ176019	USA	2001	1	Typical	92	EU807840	China	2008	8	HP
2	DQ176020	USA	2001	1	Typical	93	EU880431	China	2008	8	HP
3	JX138233	Korea	2007	1	Typical	94	EU880435	China	2008	8	HP
4	JN654459	USA	2008	1	Typical	95	EU880436	China	2008	8	HP
5	JN660150	USA	2008	1	Typical	96	FJ556871	China	2008	8	HP
6	JX138234	Korea	2009	2	Typical	97	FJ889129	China	2008	8	HP
7	AY881994	China	2005	3	Typical	98	FJ889130	China	2008	8	HP
8	JX138236	Korea	2010	3	Typical	99	GQ359108	China	2008	8	HP
9	KC771288	Korea	2013	3	Typical	100	GQ374442	China	2008	8	HP
10	AB288356	Japan	1992	4	Typical	101	GQ499193	China	2008	8	HP
11	AF176348	Canada	1995	5	Typical	102	GQ499194	China	2008	8	HP
12	AF331831	China	1996	5	Typical	103	GQ499195	China	2008	8	HP
13	AY585241	Korea	1997	5	Typical	104	GQ499196	China	2008	8	HP
14	AY612613	Korea	1997	5	Typical	105	GU168569	China	2008	8	HP
15	AF046869	USA	1997	5	Typical	106	GU169411	China	2008	8	HP
16	DQ459471	China	1998	5	Typical	107	GU232735	China	2008	8	HP
17	DQ056373	Thailand	2001	5	Typical	108	GU232738	China	2008	8	HP
18	EU880441	China	2002	5	Typical	109	HM016158	China	2008	8	HP
19	DQ473474	Korea	2002	5	Typical	110	HM853673	China	2008	8	HP
20	AY457635	China	2003	5	Typical	111	HQ315836	China	2008	8	HP
21	FJ175689	China	2003	5	Typical	112	FJ895329	China	2009	8	HP
22	FJ899592	China	2003	5	Typical	113	GQ857656	China	2009	8	HP
23	EU880443	China	2004	5	Typical	114	GU168567	China	2009	8	HP
24	DQ246451	China	2005	5	Typical	115	GU168568	China	2009	8	HP
25	EF153486	China	2006	5	Typical	116	HM016159	China	2009	8	HP

No.	GenBank[a]	Country of origin	Year of isolation	Classification		No.	GenBank[a]	Country of origin	Year of isolation	Classification	
				Lineage[b]	*Clade*[c]					*Lineage*[b]	*Clade*[c]
26	FJ194950	Korea	2006	5	Typical	117	HM189676	China	2009	8	HP
27	JN864948	China	2007	5	Typical	118	HM214913	China	2009	8	HP
28	JX138235	Korea	2007	5	Typical	119	HM214914	China	2009	8	HP
29	GQ914997	China	2009	5	Typical	120	HM214915	China	2009	8	HP
30	JQ087873	USA	2010	5	Typical	121	HQ315835	China	2009	8	HP
31	KC771287	Korea	2013	5	Typical	122	HQ315837	China	2009	8	HP
32	AY032626	China	1996	8	HP*	123	HQ843178	China	2009	8	HP
33	JN654458	USA	1996	8	Typical	124	HQ843179	China	2009	8	HP
34	AF325691	USA	1997	8	Typical	125	HQ843180	China	2009	8	HP
35	AY545985	USA	1997	8	Typical	126	HQ843181	China	2009	8	HP
36	AY150312	China	2002	8	HP*	127	JF268672	China	2009	8	HP
37	AY262352	China	2002	8	HP*	128	JF268673	China	2009	8	HP
38	EU880438	China	2002	8	HP*	129	JF268674	China	2009	8	HP
39	HQ233605	China	2002	8	HP*	130	JF268675	China	2009	8	HP
40	FJ536165	China	2004	8	HP*	131	JF268676	China	2009	8	HP
41	EU864232	China	2005	8	HP*	132	JF268677	China	2009	8	HP
42	EF075945	China	2006	8	HP	133	JF268678	China	2009	8	HP
43	EF112445	China	2006	8	HP	134	JF268679	China	2009	8	HP
44	EF112446	China	2006	8	HP	135	JF268680	China	2009	8	HP
45	EF112447	China	2006	8	HP	136	JF268681	China	2009	8	HP
46	EF517962	China	2006	8	HP	137	JF268682	China	2009	8	HP
47	EF635006	China	2006	8	HP	138	JF268683	China	2009	8	HP
48	EF641008	China	2006	8	HP	139	JF268684	China	2009	8	HP
49	EU097706	China	2006	8	HP	140	JF748717	China	2009	8	HP
50	EU097707	China	2006	8	HP	141	JF800911	China	2009	8	HP
51	EU106888	China	2006	8	HP	142	JF748718	China	2010	8	HP
52	EU109502	China	2006	8	HP	143	JF796180	China	2010	8	HP
53	EU109503	China	2006	8	HP	144	JN387272	China	2010	8	HP
54	EU144079	China	2006	8	HP	145	JN387273	China	2010	8	HP
55	EU708726	China	2006	8	HP	146	JQ663540	China	2010	8	HP

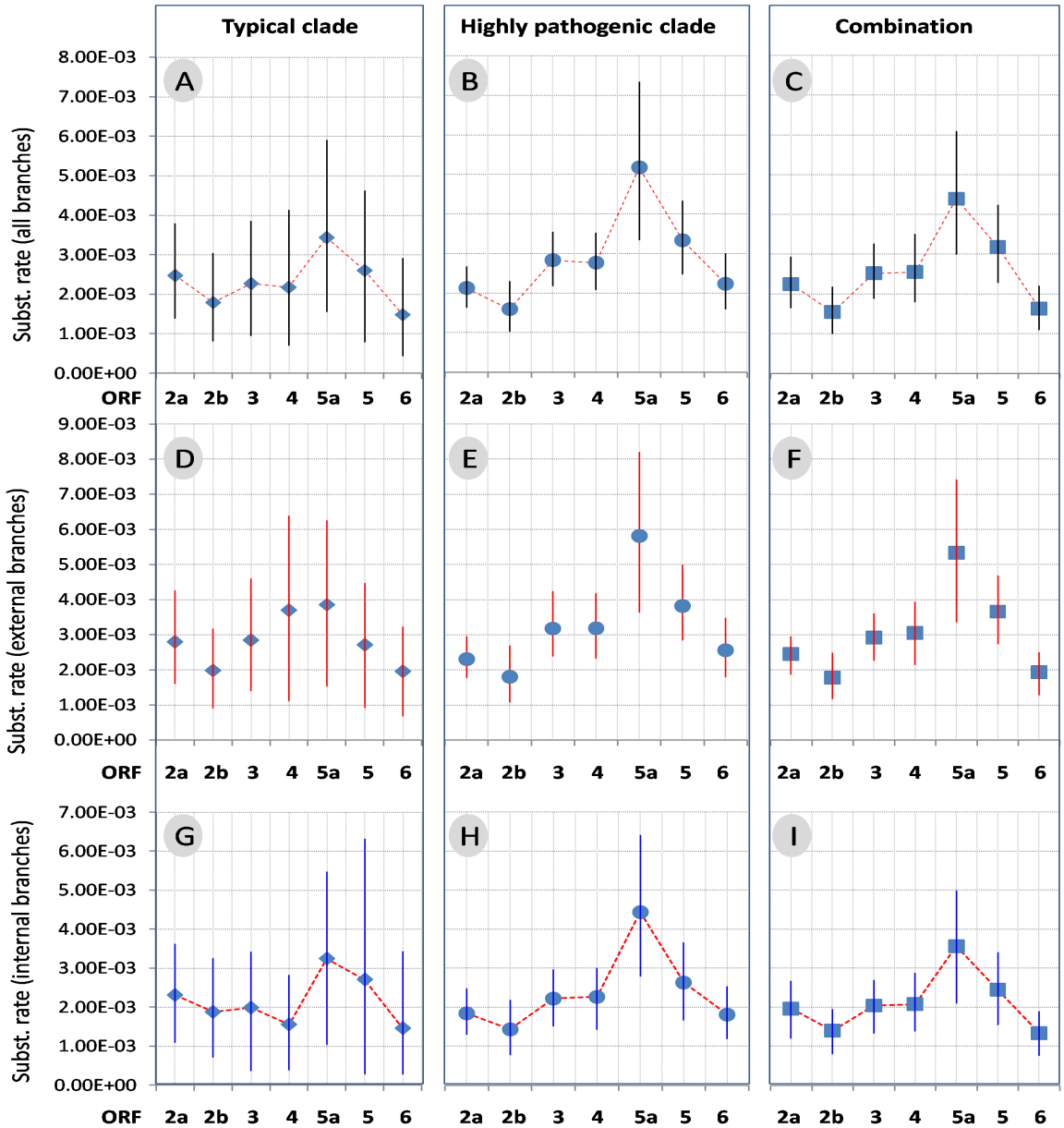
No.	GenBank[a]	Country of origin	Year of isolation	Classification		No.	GenBank[a]	Country of origin	Year of isolation	Classification	
				Lineage[b]	*Clade*[c]					*Lineage*[b]	*Clade*[c]
56	EU860248	China	2006	8	HP	147	JQ663541	China	2010	8	HP
57	EU864233	China	2006	8	HP	148	JQ663542	China	2010	8	HP
58	EU880432	China	2006	8	HP	149	JQ663543	China	2010	8	HP
59	EU939312	China	2006	8	HP	150	JQ663544	China	2010	8	HP
60	FJ797690	China	2006	8	HP	151	JQ663545	China	2010	8	HP
61	HQ233604	China	2006	8	HP	152	JQ663546	China	2010	8	HP
62	EF488048	China	2007	8	HP	153	JQ663547	China	2010	8	HP
63	EU187484	China	2007	8	HP	154	JQ663548	China	2010	8	HP
64	EU200961	China	2007	8	HP	155	JQ663549	China	2010	8	HP
65	EU200962	China	2007	8	HP	156	JQ663550	China	2010	8	HP
66	EU236259	China	2007	8	HP	157	JQ663551	China	2010	8	HP
67	EU262603	China	2007	8	HP	158	JQ663552	China	2010	8	HP
68	EU624117	China	2007	8	HP	159	JQ663553	China	2010	8	HP
69	EU825723	China	2007	8	HP	160	JQ663555	China	2010	8	HP
70	EU825724	China	2007	8	HP	161	JQ663556	China	2010	8	HP
71	EU860249	China	2007	8	HP	162	JQ663557	China	2010	8	HP
72	EU864231	China	2007	8	HP	163	JQ663558	China	2010	8	HP
73	EU880433	China	2007	8	HP	164	JQ663559	China	2010	8	HP
74	EU880434	China	2007	8	HP	165	JQ663560	China	2010	8	HP
75	EU880437	China	2007	8	HP	166	JQ663561	China	2010	8	HP
76	FJ393456	China	2007	8	HP	167	JQ663562	China	2010	8	HP
77	FJ393457	China	2007	8	HP	168	JQ663563	China	2010	8	HP
78	FJ393458	China	2007	8	HP	169	JQ663564	China	2010	8	HP
79	FJ950745	China	2007	8	HP	170	JQ663565	China	2010	8	HP
80	FJ950746	China	2007	8	HP	171	JQ663566	China	2010	8	HP
81	FJ950747	China	2007	8	HP	172	JX317649	China	2010	8	HP
82	GQ351601	China	2007	8	HP	173	JN626287	Laos	2010	8	HP
83	GQ374441	China	2007	8	HP	174	JN387274	China	2011	8	HP
84	GU454850	China	2007	8	HP	175	JQ326271	China	2011	8	HP
85	GU461292	China	2007	8	HP	176	JQ715697	China	2011	8	HP

No.	GenBank[a]	Country of origin	Year of isolation	Classification		No.	GenBank[a]	Country of origin	Year of isolation	Classification	
				Lineage[b]	*Clade*[c]					*Lineage*[b]	*Clade*[c]
86	HM011104	China	2007	8	HP	177	JQ715698	China	2011	8	HP
87	HQ401282	China	2007	8	HP	178	JQ663554	China	2012	8	HP
88	JN387271	China	2007	8	HP	179	JX087437	China	2012	8	HP
89	JX317648	China	2007	8	HP	180	JX177644	China	2012	8	HP
90	FJ394029	Vietnam	2007	8	HP	181	HQ699067	USA	2006	9	Typical
91	EU678352	China	2008	8	HP						

a) Sequences downloaded from GenBank. The two sequences (KC771287, KC771288) were generated in this study.

b) Type 2 PRRSV was classified into lineages 1 to 9 (Shi, et al., 2010)

c) Type 2 PRRSV was divided into the highly pathogenic clade (HP) which contained sequences closely clustered with the highly pathogenic type 2 PRRSV that were identified in China in 2006 (Tian et al., 2007) and its putative common ancestor (HP). The remaining type 2 PRRSV strains were classified as the typical clade.*



Supplementary Figure S3.1. Nucleotide evolutionary rates of the typical clade, the highly pathogenic clade of type 2 PRRSV, and the combination of both clades. The rates (substitutions/site/year) are measured for all branches (A-C), external (D-F) and internal (G-I) branches.

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